1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1992	THESIS/~~DISSERTATION~~

4. TITLE AND SUBTITLE
An Assessment of a Rapid Microwave Thawing Procedure for Frozen Red Blood Cells

5. FUNDING NUMBERS

6. AUTHOR(S)
Dianne C. Davis, Captain

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
AFIT Student Attending: Bowling Green State University

8. PERFORMING ORGANIZATION REPORT NUMBER
AFIT/CI/CIA- 92-095

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
AFIT/CI
Wright-Patterson AFB OH 45433-6583

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release IAW 190-1
Distributed Unlimited
ERNEST A. HAYGOOD, Captain, USAF
Executive Officer

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS	15. NUMBER OF PAGES
	65
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

AN ASSESSMENT OF A RAPID MICROWAVE THAWING PROCEDURE FOR FROZEN RED BLOOD CELLS

Dianne C. Davis

A Thesis

Submitted to the Graduate College of Bowling Green State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 1992

Committee:

Lee A. Meserve, Advisor

Carmen F. Fioravanti

Jerry A. Holmberg

Stan L. Smith

ABSTRACT

Lee A. Meserve, Advisor

Paired units of frozen red blood cells (RBC) were thawed in a conventional 42 °C waterbath and a microwave oven in order to determine the suitability of the microwave method for thawing RBC. A structurally modified microwave oven equipped with rotating mixing arms and temperature sensors was used. Measurements of freeze-thaw-wash recovery, supernatant potassium, osmotic fragility, adenosine 5'-triphosphate and 2,3-diphosphoglycerate revealed no significant differences ($p \geq 0.05$) between the thawing methods. Microwave-thawed RBC units had significantly increased levels of supernatant hemoglobin and white blood cells. The elevated white blood cell counts artifically reflected microaggregate formation. Overall, the microwave-thawed RBC were similar to the waterbath-thawed control RBC indicating that the functional and biologic integrity of the blood was not altered by the electromagnetic radiation. Thawing time averaged one-fifth as long using the microwave oven making this thawing method an attractive, time-saving alternative to the currently used waterbath method.

ACKNOWLEDGMENTS

This research was supported by the United States Naval Department and funded by the Clinical Investigation Department of the National Naval Medical Center. All studies were conducted in the Laboratory Medicine Department, Blood Bank Division, Bethesda, MD. The opinions and conclusions contained are those of the author and are in no way to be interpreted as official or reflecting the views of the Department of the Navy. I wish to acknowledge the support, guidance, and effort extended by my Bethesda advisors, LCDRs Jerry Holmberg and Randy Slater, as well my university advisor, Dr. Lee Meserve. Sincere gratitude is also extended to my parents, Robert and Velda VanAntwerp, whose unfailing faith served as an inspiration toward the accomplishment of this milestone.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The thawing of frozen blood products to be used for therapeutic human administration is regulated by the Food and Drug Administration (FDA) and standardized by the American Association of Blood Banks (AABB). Approved thawing practices for frozen red blood cells (RBC) requires thawing in either a circulating waterbath or a dry-warmer environment maintained at 37-42 °C. These thawing methods, when used with accepted freezing protocols, result in recovery of at least 80% of the original RBC mass and viability of at least 70% of the cells 24 h after transfusion. These recovery and viability levels are now the required minima acceptable by the FDA and AABB to insure adequate therapeutic response.[1,2] Alternative thawing protocols must meet or exceed these criteria.

Currently, the most widely employed RBC thawing method is waterbath immersion. Standard practice using this thawing technique includes overwrapping the primary blood storage bag in a sealed secondary pouch prior to immersion.[3] This overwrap serves to limit possible exposure of the blood bag to bacterial contaminants inherent with waterbath systems but does not eliminate the possibility of overwrap leakage and thus contamination. Unfortunately this overwrap also creates an air-water interface detrimental to heat exchange, and this is contributory to extended thawing times.

Adherence to the current waterbath or dry-warmer thawing protocols renders existing practices both time consuming and susceptible to covert bacterial contamination. Waterbath thawing can add up to 40 min to the processing time

required to prepare an overwrapped, frozen RBC unit for transfusion.[4] This can prove life-threatening to patients in emergency situations. Of further concern is the Military Blood Program 2004 of the Department of Defense which established prepositioned frozen blood products at strategic locations for wartime contingencies.[5] Extended thawing times when the majority of available products are frozen would significantly impact the speed and effectiveness of wartime medical interventions. The thawing of large volumes of blood in short periods of time employing current methods is a virtual impossibility and seriously hinders the use of frozen blood products.

A significant benefit derived from using microwave energy to thaw a frozen blood product is the speed at which the desired temperature is reached. Warming occurs because the water and polar organic molecules contained in the material vibrate when exposed to electromagnetic waves. In microwave devices, the high frequency alternating electric field reverses 2,450 million times per sec (2.45×10^9 Hz). As a result, the polar molecules are continually reversed in the field and the resulting molecular friction generates heat. The heat in turn thaws the frozen product.[6] Because of the labile nature of blood proteins, the heat produced must be closely monitored to avoid protein denaturation.

Studies in the 1940s by Ham[7] concluded that heat applied to RBC affected the integrity of the plasma membrane and resulted in changes in morphology. An increase in osmotic and mechanical fragility of the RBC was noted and in some instances gross hemolysis occurred. Thus, for any microwave procedure to prove

advantageous when applied to frozen cellular products it would be necessary to avoid the excessive generation of heat which would result in membrane damage detrimental to cell survival and function.

To the present time, microwave studies have centered on providing warmed blood in surgical cases where large transfusion volumes were required.[8-20] Though concentrating on warming blood from refrigerated storage temperature and not on thawing from sub-zero temperatures, these studies are important in delineating possible effects of microwave treatment of RBC.

Leonard investigated microwave technology as a means to alleviate the side effects of hypothermia and cardiac arrest by providing warmed blood to patients receiving massive transfusions. In this study microwave radiation did not reduce cell viability, a conclusion drawn from the absence of significant variation in adenosine 5'-triphosphate (ATP) levels between warmed and unwarmed RBC units.[11]

Later studies reported, in some instances, an increased hemolysis in microwave-treated RBC units as evidenced by measurably increased supernatant hemoglobin (HGB) levels. Staples *et al.* investigated extracorporeal hemolysis associated with the use of microwave blood warmers and concluded that excessive hemolysis could be avoided by rotating the unit properly within the heating field. In the absence of mixing, the blood exhibited evidence of overheating including hemolysis, reduced viability, and morphological abnormalities.[12] Blood proved to be a poor heat conductor and caused the 2,450 MHz electromagnetic energy to be absorbed in the outermost layers of the unit. This contributed to overheating at the

exterior surfaces of the storage bag and caused increased hemolysis. Investigators concluded that uneven warming and elevated cellular water content inhibited penetration of electromagnetic energy.[14,18]

A red blood cell warming study examining use of a microwave oven modified with a rotating blood holder was conducted by Roth-Henschker.[20] The mechanical modification allowed mixing to promote uniform warming and additionally incorporated an automatic temperature monitoring device. Use of this microwave oven allowed warming of RBC to 32 °C with no observable effect if small volumes, hematocrits over 70%, and increased viscosities were avoided.

Thawing trials applying microwave energy to frozen blood products were first conducted on cell-free frozen plasma.[21-29] Mead used a combination of tapwater immersion and standard microwave oven processing to achieve a 33% decrease in thaw time without detriment to coagulation factor function.[21] The drawback of possible contamination with water through the port remained when using this procedure.

Studies by Checcucci[22] and Rock[23] successfully employed direct microwave processing without a water interface. The later study resulted in the development of a structurally modified microwave oven equipped with a rotating mixing arm and a temperature sensing shut-off device. These studies established microwave thawing of frozen plasma as an acceptable alternative and led to mechanical modification of microwave ovens for this purpose. The FDA subsequently approved a modified microwave oven equipped with rotating chambers solely for plasma thawing.[30]

Few investigations have specifically addressed frozen RBC thawing using microwave energy and only limited information is available to suggest that this could be a successful thawing technique.[31-35] Slater examined the use of an unmodified carousel microwave oven and frozen RBC immersed in a heat-dispersing medium such as sand, glycerol, or tap water. In this study, microwave-processed RBC units thawed 5 times faster than control RBC units thawed in a waterbath. Red blood cell integrity was well-maintained as evidenced by stable ATP and 2,3-diphosphoglycerate (DPG) levels. An elevation in supernatant HGB was noted in the units subjected to microwave thawing, but was deemed clinically insignificant.[35]

Ideally, if a microwave thawing protocol was proven equivalent to the waterbath thawing protocols in producing an acceptable transfusion product, then it could replace current time-consuming practices. Benefits foreseen from microwave thawing of frozen RBC include both time savings per unit processed and the elimination of the threat of bacterial contamination. The dual use of a single thawing apparatus for frozen plasma and RBC eliminates duplication of equipment and technician error associated with performing multiple methodologies. Budgetary and workplace spacial constraints also encourage employment of a single thawing apparatus. The current study was formulated to assess the impact of rapid microwave thawing of frozen RBC using the structurally modified microwave plasma defroster approved by the FDA. The investigational design centered on comparing recovery and viability in microwave-thawed RBC vs standard heat-transfer waterbath-thawed RBC.

MATERIALS AND METHODS

Baseline Pooling, Sampling, and Testing

Studies were undertaken on 6 pools of volunteer donor blood. Each of the pools was composed of 8 fresh units, within 7 days of collection, of packed RBC which had been tested and found suitable for transfusion in accordance with FDA regulations[36] and AABB standards.[1,2] Each donor unit contained approximately 450 mL of whole blood collected into an 800 mL quadruple plastic bag system (Fenwal Laboratories, Deerfield, IL) containing 63 mL of citrate-phosphate-dextrose-adenine anticoagulant. The whole blood units were processed to produce packed RBC units which were then stored at 2-6 °C until pooled. Donor blood collection and unit processing were performed by personnel assigned to the Blood Donor Section, National Naval Medical Center, Bethesda, MD.

For each pool, 8 units of group-identical packed RBC were combined in a RCM-3 biological storage bag (Stericon, Inc., Broadview, IL) using an automated sterile docking device (Haemonetics Corporation, Braintree, MA). Prior to pooling all units were tested and found to be hemoglobin S negative (Appendix A-1).[37] Each pool was thoroughly mixed by manual agitation.

A 10 mL sample was obtained from each pool by accessing the pool storage bag through a sample site coupler (Fenwal Laboratories, Deerfield, IL) placed in a port and using an 18 gauge needle and syringe assembly (Becton, Dickenson, & Company, Columbus, NE). This sample was stored at 2-6 °C in a capped plastic tube

(Superior Healthcare Group, Inc., Cumberland, RI) and analyzed for baseline values within 4 h of pooling. Baseline testing included an automated complete blood count (CBC) to establish pool levels for RBC, white blood cells (WBC), HGB, hematocrit (HCT), and mean corpuscular volume (MCV). Baseline testing also established prefreeze concentrations of ATP and DPG (Appendix A).

Each pool was then thoroughly re-mixed and divided into eight 250 g aliquots using a Lume-o-gram balance (Ohaus Scale Corporation, Florham Park, NJ) and sterile docking between the pooling bag and the original 800 mL primary collection bag. The aliquots were returned to the original primary bags for glycerolization and freezing. The high glycerol (40%), low temperature (-65 °C) freezing and deglycerolization protocol established by the Naval Blood Research Laboratory (NBRL) was followed for all RBC units included in the study.[4] In each pool, 4 of the 8 RBC units were randomly designated waterbath controls and the remaining 4 units were designated the microwave treatment group.

Thawing Methods

After freezing and storage at or below -65 °C, the individual RBC units were thawed either conventionally in a 42 °C waterbath or in the FDA-approved microwave plasma defroster. Paired waterbath control RBC units and microwave-treated RBC units were thawed simultaneously.

All waterbath control RBC units were thawed in a covered, thermostatically controlled, M20 Lauda circulating waterbath (Wobser GMBH & Company,

Konigshofen, Germany). The water temperature was confirmed independent of waterbath controls using a National Bureau of Standards (NBS) certified thermometer. The circulating pump was engaged for 5 min prior to frozen unit immersion and each unit was processed individually. Each waterbath control unit was enclosed in a sealed plastic protective overwrap.[3] The RBC unit was counter-weighted to ensure total submersion during thawing and was examined at 5 min intervals. Maximum allowable waterbath immersion time was 40 min as recommended by the thawing protocol.[4]

Microwave thawing was accomplished in the WesLabs Plasma Defroster (Westmorland Laboratories Inc., New Brunswick, Canada).[30] These units of packed RBC were not overwrapped and no more than 1 unit was placed in the microwave oven at a time. Each RBC unit was positioned in a metal bag-holder with the label side away from the temperature sensing probe position. The bag-holder was attached to 1 of the 4 shafts in the thawing chamber. During thawing, the bag-holder containing the frozen RBC unit underwent rotational oscillation to avoid continuous, concentrated microwave exposure. The temperature sensory probe of the microwave oven adjusted the microwave energy to appropriate levels during the thawing cycle and automatically shut down. Cycle completion was programmed to occur when the sensing probe reached approximately 23 °C and was non-adjustable. Because the automated cycle was set for plasma stored at no colder than -50 °C, the cycling for the frozen RBC units stored at or below -65 °C was monitored continuously to insure cycle completion. Each RBC unit was manually checked for complete thawing at

cycle completion and restarted if necessary.

Physical Appearance

The post-thaw physical appearance of each unit of packed RBC was examined for evidence of discoloration, cellular aggregation, or unusual viscosity. Any evidence of overheating was noted.

Thaw Time

The time interval from initial RBC unit immersion or the start of the microwave cycle to the thawed endpoint was measured using a calibrated laboratory timer (West Bend Company, West Bend, WI).

Temperature Measurement

Unit core temperature measurement post-thaw was taken using an electronic YSI Tele-Thermometer with 400 series probe (Fisher Scientific, Pittsburgh, PA). The tele-thermometer was calibrated against a NBS certified thermometer. The probe was fully inserted into each unit of RBC via a transfusion port immediately after removal from the thawing apparatus. The unit was manually mixed and the temperature recorded at 1 min post probe insertion. The accessed port was aseptically sealed upon probe withdrawal with a sterile sample site coupler (Fenwal Laboratories, Deerfield, IL).

Post-Thaw Sampling

After deglycerolization each RBC unit was weighed using the Lume-o-gram balance and then aseptically sampled. Samples were collected immediately post-wash and after 24, 48, and 72 h of storage for the following tests: osmolality, supernatant HGB and potassium, CBC, osmotic fragility, ATP, and DPG. All measurements were performed in duplicate on each unit at each interval. Freeze-thaw-wash recoveries were calculated as described below. Units were stored in a 2-6 °C monitored refrigerator through the 72 h test interval.

Sampling was performed by inserting an 18 gauge needle attached to a 20 mL syringe (Becton, Dickenson, & Company, Columbus, NE) through the sample site coupler (Fenwal Laboratories, Deerfield, IL) at the port access. A 20 mL sample was withdrawn at each test interval from each waterbath-thawed and microwave-thawed RBC unit. To avoid mechanical hemolysis the needle was removed from the syringe prior to dividing the 20 mL sample into 2 equal 10 mL portions. The divided samples from each RBC unit were stored at 2-6 °C in appropriately labeled, capped plastic tubes (Superior Healthcare Group, Inc., Cumberland, RI) and tested within 4 h of sampling.

For each unit of RBC, 1 of the 10 mL samples was separated into packed RBC and supernatant using a Dynac II centrifuge (Becton, Dickenson, & Company, Columbus, NE). The timed separation period was 10 min at 2500 rpm (1150 x g). The supernatant obtained was used to determine osmolality, and HGB and potassium levels. The remaining packed RBC volume was used to produce smears on glass

slides (Erie Scientific Company, Portsmouth, NH) for morphological examination. The second, uncentrifuged 10 mL sample was used to perform CBC, osmotic fragility, ATP, and DPG testing.

Freeze-Thaw-Wash Red Blood Cell Recovery

Percentage recovery of red blood cells was calculated using both the standard AABB[2] and NBRL[4] formulas.

AABB Formula:

$$\text{Recovery} = \frac{\text{Net weight (g) of deglycerolized RBC X HCT}}{\text{Net weight (g) of prefreeze RBC X HCT}}$$

Note: Multiply value by 100 for percent.

NBRL Formula:

The NBRL formula uses the total cellular HGB contained in each unit based on density, both pre-freeze and post-deglycerolization. Steps 1, 2, and 3 below:

1. Density Formula:

$$\text{Prefreeze unit} = \frac{1.1 + (\text{HCT in decimal form} - 0.20)}{10}$$

$$\text{Deglycerolized unit} = \frac{1.0 + (\text{HCT in decimal form} - 0.05)}{10}$$

Note: The constant factors 1.1 and 1.0 are the densities of the prefreeze plasma and deglycerolized isotonic suspension fluids, respectively. The remaining constants, 0.20 and 0.05, are standard factors for the supernatant content within the RBC, pre-glycerolization and post-deglycerolization.

2. Unit Hemoglobin Formula:

$$\text{HGB (g)} = \frac{(\text{HGB X Net weight (g)}) \text{ divided by Density}}{100}$$

3. $$\% \text{ Recovery} = \frac{\text{Grams HGB in Deglycerolized Unit}}{\text{Grams HGB in Prefreeze Unit}} \text{ X } 100$$

Both methods of calculating recovery used the net weight of the unit determined by subtracting the weight of the blood bag from the gross weight of the bag plus the RBC component. The unit of measurement was grams (g).

Morphology Studies

A smear was made, Wright-stained, examined, and photographed under oil immersion (magnification x20, x40, and x100) for each RBC unit immediately post-wash and after 24, 48, and 72 h of storage. An Olympus model BH2 Microscope with C35AD-4 camera attachment was used (Olympus, Tokyo, Japan). Visual, qualitative interpretation of gross cellular morphology was made.

Osmolality

Osmolality was determined as a quality control measure to insure adequate glycerol removal. The principle of freezing point depression was employed with a Model 3DII osmometer (Advanced Instruments, Inc., Needham Heights, MA) (Appendix A-2).[38] Upper permissible limit by AABB standards[1,2] is 500 mOsm and all units included in the final study were acceptable by this criterion.

Waste and Supernatant Hemoglobin

Hemoglobin concentration was measured in the waste from the deglycerolization procedure and in the unit supernatant. These values were used as indicators of RBC membrane damage and subsequent cellular lysis. Levels were measured using a direct spectrophotometric procedure (Appendix A-3).[39] Wash waste HGB levels were determined immediately post-deglycerolization and the unit supernatant HGB levels were determined immediately post-wash and after 24, 48, and 72 h of storage for each RBC unit.

Supernatant Potassium

Supernatant potassium concentrations were determined with an ion selective electrode by comparison against a known calibration standard (Astra 8 System, Fullerton, CA) (Appendix A-4).[40] Values were used as indicators of RBC membrane damage and subsequent cellular lysis. Concentrations of potassium were determined on the supernatant of each RBC unit immediately post-wash and after 24, 48, and 72 h of storage.

Complete Blood Count

The RBC count, WBC count, HGB, HCT, and MCV were determined for each unit using a Coulter S-Plus III series instrument (Coulter Electronics, Hialeah, FL) (Appendix A-5).[41] A prefreeze baseline determination was established for each RBC unit as were post-deglycerolization measurements immediately post-wash and

after 24, 48, and 72 h of storage.

Osmotic Fragility

Osmotic fragility was used to determine RBC membrane leakage and instability induced by either waterbath or microwave oven thawing. Osmotic fragility was determined by suspending the thawed, washed RBC in concentrations of sodium chloride (NaCl) varying from 0.00% to 0.85% and spectrophotometrically measuring the amount of HGB released (Appendix A-6).[42] Measurements for each unit were made immediately post-wash and after 24, 48, and 72 h of storage.

Adenosine 5'-Triphosphate

Post-transfusion RBC survival has been found to be related primarily to ATP concentration.[43-52] Values were determined using a kit (Catalog #366-A, Sigma Diagnostics, St. Louis, MO) for quantitative enzymatic determination based on spectrophotometrically measurable changes in absorbance (Appendix A-7).[53] A standard of known ATP concentration (Catalog #A5394, Sigma Diagnostics, St. Louis, MO) was assayed with each test series to insure accurate measurement. A prefreeze baseline level was established for each RBC unit as were post-deglycerolization levels immediately post-wash and after 24, 48, and 72 h of storage.

2,3-Diphosphoglycerate

Maintenance of oxygen-transport functions in RBC has been determined to

depend primarily on the concentration of DPG present in the cells.[46-52,54] Levels were determined using a kit (Catalog #35-A, Sigma Diagnostics, St. Louis, MO) for quantitative enzymatic determination based on spectrophotometrically measurable changes in absorbance (Appendix A-8).[55] A standard of known DPG concentration (Metabolite Control #53005, Sigma Diagnostics, St. Louis, MO) was assayed with each test series to insure accurate measurement. A prefreeze baseline level was established for each RBC unit as were post-deglycerolization levels immediately post-wash and after 24, 48, and 72 h of storage.

Statistical Analysis

Analyses of variance (ANOVA) for mixed models with crossed and nested factors were performed on test results using Minitab Statistical Software Release Numbers 7.1 and 8.0 (Minitab Inc., State College, PA).[56] Analyses calculated means, F-tests, and p values for all data across the testing time interval, for pools, and for treatment type. Interaction factors were calculated for all combinations of time, pools, and treatments (waterbath and microwave). Significance was ascribed to any comparison with a p value less than or equal to 0.05.

RESULTS

The present study was undertaken to examine the feasibility of using the FDA-approved microwave plasma defroster to thaw frozen RBC. Successful application would decrease both processing time and the threat of bacterial contamination. The investigational focus was centered on evaluating microwave-thawed units for similar cellular recovery and viability relative to conventional waterbath-thawed units.

A pre-study pilot was performed on unpooled, expired frozen RBC to identify spacial constraints within the microwave bag-holder. The pilot study lead to a modification of the standard NBRL freezing protocol, eliminating the fold in the storage bag. Accordingly, the 24 microwave units in the main body of the study were frozen without folding; the 23 waterbath units were folded as usual. One waterbath control unit was eliminated from the study owing to mechanical failure along a bag seamline during the glycerolization procedure.

The physical appearance of the majority of RBC units after thawing was unremarkable. Visually, there were no discernible signs of hemolysis, discoloration, aggregates, or increased viscosity in any waterbath control unit examined. Six of 24 microwave-thawed RBC units (25%) appeared to contain small chocolate brown microaggregates. These were confined along the bag seamlines and at the access ports.

All waterbath control units required at least the maximum allowable 40 min at 42 °C to achieve a temperature acceptable for deglycerolization. Additionally, 7 of these units (30%) required additional room temperature (RT) incubation to achieve a

core temperature above the required 20 °C. Thaw times for the waterbath units ranged from 40 to 70 min with a mean of 46 min. The range of thaw times for microwave units was from 5 min 39 sec to 12 min 20 sec. The microwave mean thaw time was 9 min. Thaw times for the 2 groups, waterbath and microwave, were significantly different when compared by the paired t-test (Table 1).

The mean core temperature of the waterbath control RBC units after 40 min of waterbath immersion was 24.1 °C with a range of 13.1-35.4 °C. The microwave-thawed RBC had a mean temperature of 29.0 °C after completing the automatic thawing cycle. The temperature range for the microwave-thawed group was 20.0-52.3 °C. Two of the microwave units recorded abnormally high core temperatures even though their processing times were comparable to the other units in the treatment group. The mean core temperatures for the 2 thawing methods were not significantly different (Table 2).

No significant difference in RBC recovery between the 2 treatment groups was found (Tables 2,3). Red blood cell recovery using the AABB calculation yielded a mean recovery of 84.4% for the waterbath group and 82.9% for the microwave group. Cellular recoveries using NBRL calculations yielded mean values of 93.4% vs 92.0% for the waterbath-thawed and microwave-thawed groups respectively. It was found that the NBRL recovery calculation on each unit was consistently about 9% greater than the AABB calculation, with mean values for waterbath +9.0% and for microwave +9.1%.

Three waterbath units (13%, n=23) and 4 microwave units (16.7%, n=24)

TABLE 1. Thaw Time of Frozen Packed RBC
Waterbath versus Microwave Thawing Methods

	Thawing Method	
	Waterbath	Microwave
Unit #	40 Minutes at 42°C /Additional RT Incubation	Automatic Shutoff Minutes/Seconds
1	40/00	10/45
2	40/00	7/40
3	40/00	9/12
4	40/00	12/20
5	40/00	7/15
6	40/00	12/07
7	40/00	7/20
8	40/00	5/39
9	*40/30	7/11
10	*40/20	6/42
11	40/00	12/15
12	#	11/15
13	*40/20	8/00
14	40/00	9/20
15	40/00	10/04
16	*40/20	10/30
17	40/00	7/33
18	40/00	10/48
19	40/00	7/30
20	40/00	11/19
21	40/00	9/40
22	*40/10	6/55
23	*40/10	7/00
24	*40/20	7/00
	Waterbath	Microwave
Range:	40 - 70 Min	5/39 - 12/20 Min/Sec
Mean ± SD	46 Min ± 9 Min 30 Sec	9 Min ± 2 Min 2 Sec

* Waterbath units requiring additional room temperature (RT) incubation.

Unit broken during processing.

SD=Standard Deviation

TABLE 2. Core Temperature and AABB Recovery
Waterbath and Microwave-Thawed RBC Units

	Thawing Method			
	Waterbath		Microwave	
Unit Number	Temp ℃	AABB Recovery(%)	Temp ℃	AABB Recovery(%)
1	24.2	82.4	37.2	87.7
2	27.1	91.0	⇑52.3	*62.9
3	28.2	85.1	29.4	82.1
4	35.4	86.2	20.7	82.1
5	25.7	93.2	24.8	*69.1
6	29.0	86.5	20.0	87.5
7	26.2	82.2	29.7	95.3
8	22.9	86.6	30.2	81.5
9	13.8	*67.3	30.5	81.9
10	13.1	82.8	29.0	83.0
11	20.6	86.3	22.8	89.9
12	#	#	20.5	87.3
13	17.5	*78.4	32.0	88.1
14	23.3	81.9	20.0	*75.6
15	25.2	83.5	⇑48.8	84.1
16	17.1	80.2	20.0	80.9
17	28.1	88.5	26.8	82.6
18	29.5	91.3	24.3	86.6
19	29.5	91.0	32.9	85.7
20	32.3	85.0	24.2	82.9
21	30.5	86.1	21.6	*74.9
22	19.1	87.6	31.6	86.0
23	19.0	*78.3	32.0	84.8
24	15.8	80.6	33.5	87.5
Mean	24.1	84.4	29.0	82.9
SD	±6.1	±5.5	±8.4	±6.8

Mean Core Temperatures and RBC Recoveries do not differ significantly regardless of thawing method ($p \geq 0.05$).

* Unacceptable Recovery (less than 80%)
\# Unit broken during processing
⇑ Abnormal core temperature

TABLE 3. AABB and NBRL Red Blood Cell Recovery
Waterbath and Microwave-Thawed Units

Freeze-Thaw-Wash Recovery (%)	Waterbath n=23	Microwave n=24
AABB Mean	84.4	82.9
SD	5.5	6.8
SE	1.2	1.4
Range	67.3-93.2	62.9-95.3
p value=0.42	NS	NS
NBRL Mean	93.4	92.0
SD	6.5	8.0
SE	1.4	1.6
Range	74.9-104.0	68.0-107.4
p value=0.53	NS	NS
Mean Difference	+9.0	+9.1

SD=Standard Deviation
SE=Standard Error
NS=Not Statistically Significant

failed to achieve the required 80% RBC recovery (Table 2). The RBC units with poor recoveries in the waterbath treatment group were those requiring additional RT incubation to reach an acceptable deglycerolization temperature. No common factor was evident in the microwave-thawed units with poor recovery.

Microscopic examination of the RBC smears made over the 72 h storage period on each unit revealed an increase in cell size in both waterbath-thawed and microwave-thawed samples. Increases in the 2 treatment groups were similar. The empirical observation was confirmed by automated MCV measurements which registered the same pattern of cell size increase over the test period. The only visible anomaly noted by microscopic observation was the appearance of rare, small, cell clusters. These clusters appeared consistently on smears, from immediately post-thaw through 72 h of storage, from the microwave units previously noted to contain microaggregates at the time of thawing. The clusters consisted of spherical cells (spherocytes), disc-shaped cells (ovalocytes), and fragmented cells (schistocytes) not noted in adjacent unaggregated areas of the slide.

Osmolality for all units ranged from 300-346 mOsm and all units were acceptable for further evaluation. There was no significant mean difference (313 vs 316 mOsm, respectively) between control and microwave units.

There was no significant difference in the HGB concentrations found in the deglycerolization waste for waterbath- or microwave-thawed units. Since this was the case, the percentage of hemoglobin lost from each unit was calculated and then used to validate and compare AABB and NBRL recovery calculations. Mathematically, the

percentage of lost RBC and the percentage of recovered RBC for each unit should approximate, within reasonable test limitations, the original starting cell mass of the unit. When AABB recovery figures were used the additive total of the 2 percentages closely reflected 100% of the original unit mass. Recovery percentages using NBRL calculations were found to yield totals in excess of the original total red blood cell mass. For example, if the mean 15.2% waste hemoglobin was added to the mean calculated waterbath recovery, AABB 84.4 vs NBRL 93.4, the total original cell mass would be 99.6% vs 108.6% respectively.

Supernatant HGB and potassium concentrations were measured for both waterbath-thawed and microwave-thawed RBC units immediately following washing and after 24, 48, and 72 h of storage (Fig 1,2). Hemoglobin levels rose markedly over the storage period for both waterbath and microwave RBC units. The same pattern of increasing concentration over time was seen in supernatant potassium values. There was a significant difference in the supernatant HGB levels between the 2 thaw groups. Microwave-thawed units demonstrated initially greater concentrations of HGB which remained elevated above control levels throughout the storage period. In contrast, concentrations of supernatant potassium varied little between the microwave- and waterbath-thawed RBC. Statistically there was no difference for this analyte between the 2 treatment groups.

Automated complete blood counts immediately post-thaw and after 24, 48, and 72 h of storage were used to monitor RBC, WBC, HGB, HCT, and MCV levels for all 47 units under investigation (Table 4). Statistical comparisons were done to

Figure 1. Comparison of supernatant hemoglobin concentration (mg/dL) in waterbath-thawed (n=23, solid bars) versus microwave-thawed (n=24, hatched bars) red blood cells over a 72 hour post-thaw period. A significant difference was seen between the two thawing methods ($p \leq 0.05$).

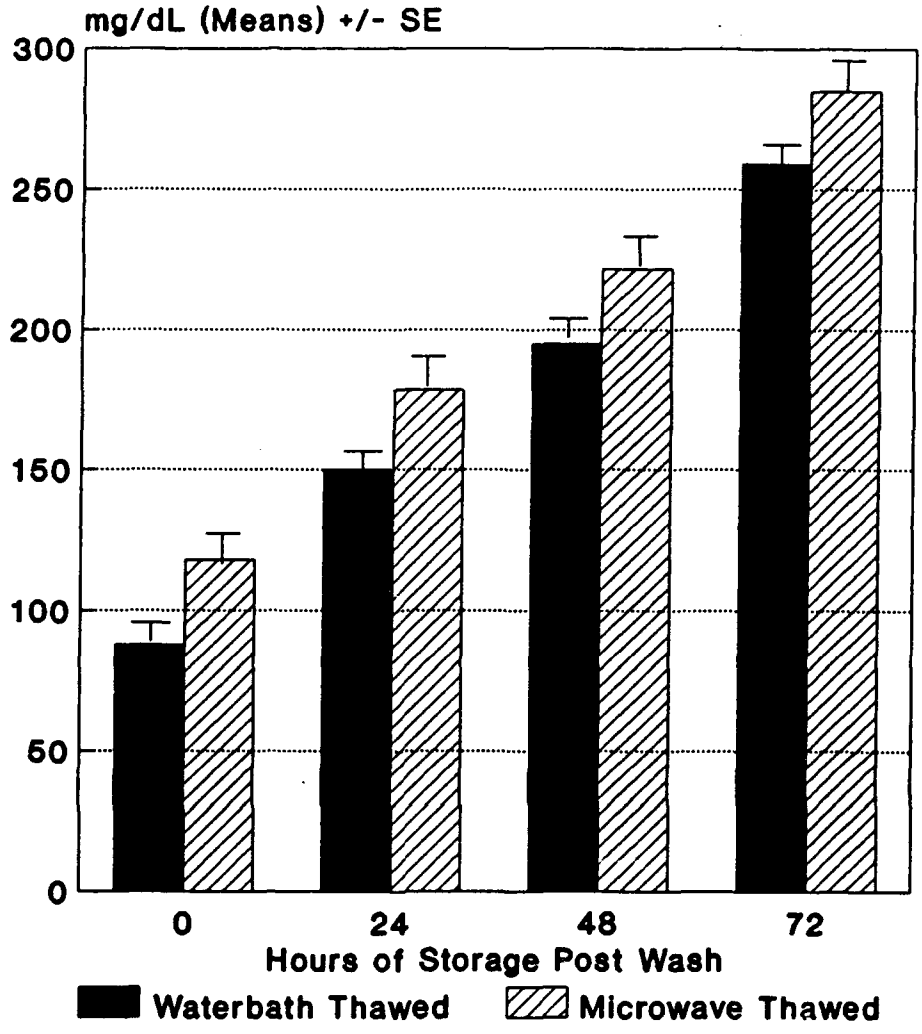

mg/dL (Means) +/- SE
300
250
200
150
100
50
0
0
24
48
72
Hours of Storage Post Wash
Waterbath Thawed
Microwave Thawed

Figure 2. Comparison of supernatant potassium concentration (mmol/L) in waterbath-thawed (n=23, solid bars) versus microwave-thawed (n=24, hatched bars) red blood cells over a 72 hour post-thaw period. No significant difference between thawing methods was seen ($p \geq 0.05$).

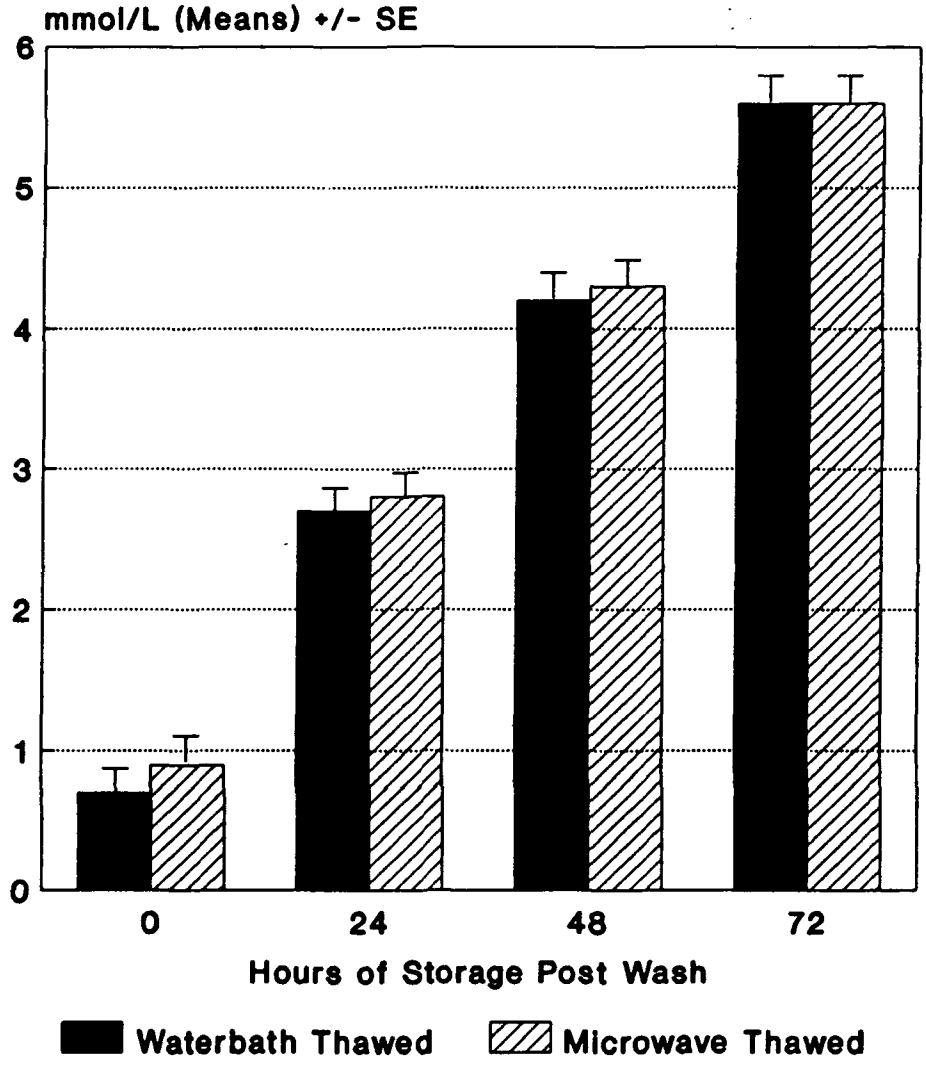

mmol/L (Means) +/- SE
6
5
4
3
2
1
0
0
24
48
72
Hours of Storage Post Wash
Waterbath Thawed
Microwave Thawed

TABLE 4. Complete Blood Count Parameters
Waterbath versus Microwave-Thawed Units

	Storage/Test Interval			
	Immediately Post Thaw	24 Hours	48 Hours	72 Hours
Red Blood Cells (X $10^6/\mu L$)				
Waterbath	3.97	3.99	4.01	4.01
Microwave	3.86	3.90	3.91	3.90
	Variance over Time p=0.71 (NS)			
	Treatment Variance p=0.19 (NS)			
	SE: Waterbath 0.03, Microwave 0.04			
White Blood Cells (X $10^3/\mu L$)				
Waterbath	0.1	0.1	0.1	0.1
Microwave	0.5	0.6	0.5	0.6
	Variance over Time p=0.39 (NS)			
	Treatment Variance p=0.01 (*S)			
	SE: Waterbath 0.01, Microwave 0.09			
Hemoglobin (g/dL)				
Waterbath	11.7	11.7	11.8	11.7
Microwave	11.5	11.5	11.5	11.5
	Variance over Time p=0.69 (NS)			
	Treatment Variance p=0.32 (NS)			
	SE: Waterbath 0.07, Microwave 0.12			
Hematocrit (%)				
Waterbath	35.7	36.3	36.5	36.7
Microwave	34.9	35.5	35.8	35.9
	Variance over Time p=0.96 (NS)			
	Treatment Variance p=0.25 (NS)			
	SE: Waterbath 0.22, Microwave 0.35			
Mean Cell Volume (fL)				
Waterbath	89.9	90.8	91.2	91.6
Microwave	90.3	91.1	91.5	92.0
	Variance over Time p=≤0.0001 (*S)			
	Treatment Variance p= 0.07 (NS)			
	SE: Waterbath 0.16, Microwave 0.16			

Waterbath Group n=23, Microwave Group n=24
NS: Not Significant ($p \geq 0.05$)
S: Significant ($p \leq 0.05$)

determine the effect of both thawing method and storage time on each assay. The RBC, WBC, HGB, and HCT levels in both waterbath and microwave units showed no significant change over the 72 h storage interval. The mean RBC count for the control units was 4.0 x $10^6/\mu L$ and 3.9 x $10^6/\mu L$ for the microwave units. WBC means were 0.1 and 0.6 x $10^3/\mu L$ for control and microwave units respectively over the 72 h storage period. The mean HGB levels were 11.7 g/dL for the waterbath control units and 11.5 g/dL for microwave-thawed units. Hemoglobin and HCT levels revealed the standard multiplicative relationship with HCT levels reflecting approximately 3 times the HGB levels.

In contrast, MCV levels in both waterbath control and microwave units rose significantly over the storage interval. The MCV of the control units increased from a mean of 89.9 fL immediately post-wash to a mean of 91.6 fL after 72 h. Microwave-thawed RBC showed a similar increase in mean MCV over the storage interval rising from 90.3 fL to 92.0 fL. Values of MCV were not significantly different when the 2 thaw methods were compared. Only 1 parameter, WBC count, exhibited a significant difference between the 2 thawing methods. WBC counts remained at means of 0.1 and 0.6 x $10^3/\mu L$ for control and microwave units respectively over the 72 h test period. The elevated WBC counts in the microwave units were attributed to the visible microaggregates seen in 7 of the 24 units tested. The remaining 17 microwave-thawed units had WBC counts that were not significantly different from the controls.

Osmotic fragility in both waterbath control RBC and microwave-thawed RBC

did not significantly change over the 72 h storage interval (Figure 3). Percent hemolysis seen at each NaCl concentration was not significantly different between units thawed using the conventional waterbath method and the experimental microwave method.

ATP concentrations for both groups of thawed RBC were measured before freezing, immediately post-wash, and after 24, 48, and 72 h of storage (Figure 4). Concentrations were similar for the 2 treatment groups, waterbath and microwave, indicating that the thawing method had no effect on the recovery of the analyte. Red blood cell ATP levels in both thawing groups were higher immediately after washing and showed a significant downward trend over the 72 h storage period. The waterbath control units immediately post-wash showed a mean level of 5.2 μmol/g or 131% of the prefreeze level. This decreased to a mean of 4.3 μmol/g or 110% of the prefreeze level by the end of the 72 h storage period. Microwave-thawed units declined from a similar post-wash mean of 5.1 μmol/g (130%) to 4.5 μmol/g (113%) after 72 h.

Concentrations of DPG were also tested at the same intervals for both treatment groups and similarly showed a significant decrease over the storage period. Values did not differ significantly between the 2 treatment groups (Figure 5). The mean value for the waterbath control units declined from a post-wash value of 7.8 μmol/g to 4.6 μmol/g after 72 h of storage. The DPG concentration for waterbath-thawed units immediately post-wash was 106% of the prefreeze level and dropped to 63% after 72 h of storage. The microwave treatment group exhibited a

Figure 3. Osmotic fragility determined by using varying concentrations of sodium chloride for waterbath-thawed (n=23, thick line) versus microwave-thawed (n=24, thin line) red blood cells. No significant difference ($p \geq 0.05$) between values for thawing methods was seen.

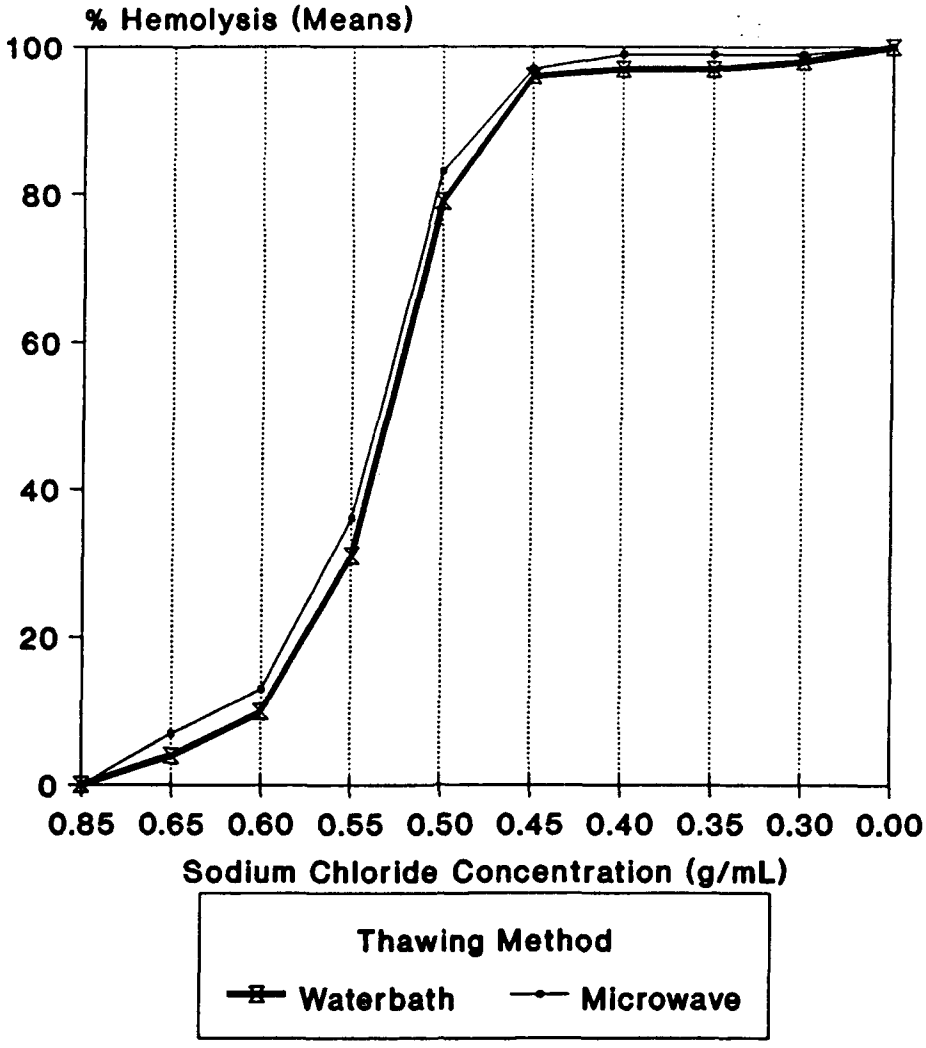
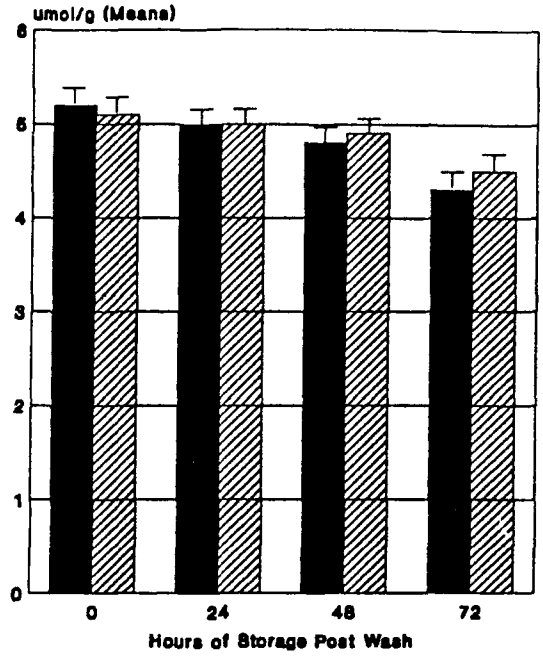

% Hemolysis (Means)
100
80
60
40
20
0
0.85 0.65 0.60 0.55 0.50 0.45 0.40 0.35 0.30 0.00
Sodium Chloride Concentration (g/mL)
Thawing Method
Waterbath
Microwave

Figure 4.

A. Comparison of Adenosine 5'-Triphosphate concentration (μmol/g) in waterbath-thawed ($n=23$, solid bars) versus microwave-thawed ($n=24$, hatched bars) red blood cells over a 72 hour post-thaw period. No significant difference between thawing methods was seen ($p \geq 0.05$).

B. Percent of prefreeze ATP recovered in waterbath-thawed (solid bars) versus microwave-thawed (hatched bars) red blood cells over a 72 hour post-thaw period.

A


umol/g (Means)
6
5
4
3
2
1
0
0
24
48
72
Hours of Storage Post Wash

B

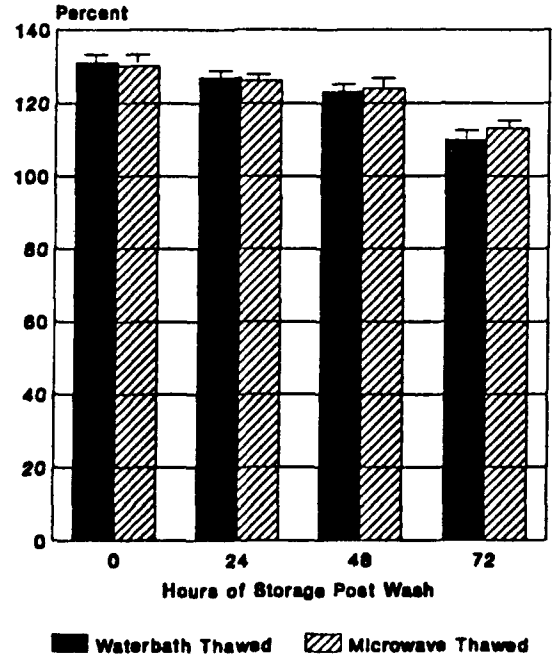
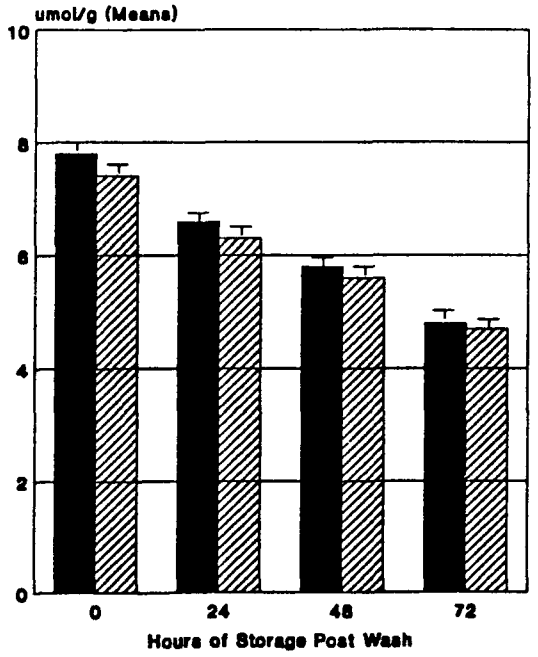

Percent
140
120
100
80
60
40
20
0
0
24
48
72
Hours of Storage Post Wash
Waterbath Thawed
Microwave Thawed

Figure 5.

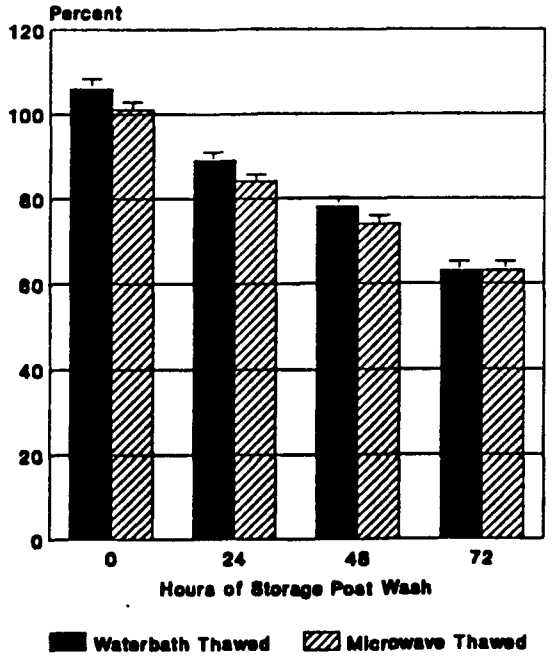
A. Comparison of 2,3-Diphosphoglycerate concentration (μmol/g) in waterbath-thawed (n=23, solid bars) versus microwave-thawed (n=24, hatched bars) red blood cells over a 72 hour post-thaw period. No significant difference between thawing methods was seen ($p \geq 0.05$).

B. Percent of prefreeze DPG recovered in waterbath-thawed (solid bars) versus microwave-thawed (hatched bars) red blood cells over a 72 hour post-thaw period.

A


umol/g (Means)
10
8
6
4
2
0
0
24
48
72
Hours of Storage Post Wash

B


Percent
120
100
80
60
40
20
0
0
24
48
72
Hours of Storage Post Wash
Waterbath Thawed
Microwave Thawed

similar mean post-wash concentration of 7.4 μmol/g (101%) which declined to 4.5 μmol/g (63%) at the end of the 72 h storage period.

DISCUSSION

Opposition to applying microwave radiation to cellular products has been focused on the concern that hot spots would develop and prevent the even distribution of the electromagnetic waves.[29] This disruption of the wave pattern would theoretically prevent uniform temperature exposure in the product and promote membrane damage in the overheated areas. The present study sought to dispel these concerns by evaluating the effect of microwave thawing on frozen RBC using controlled conditions and continuous mixing. It was anticipated that the high lipid content of the RBC membrane, with its hydrophobic qualities and low dielectric constant, would protect the membrane from significant structural damage. The hydrophilic amino acid residues contained in the underlying cytoskeleton would then facilitate warming and therefore thawing. As thawing commenced, the mixing would ensure that the liquid blood remained in contact with the moderating influence of the remaining frozen blood.

An initial concern was that the standard glycerolization protocol[4] required that a fold be placed in the primary blood bag prior to freezing. A pilot investigation resulted in a change in the bag configuration. This modification involved draining, rolling, and clipping the bottom section of the primary bag to reduced expansion space available to the liquid RBC. This eliminated visibly unequal microwave heating by eliminating the folded smaller sequestered section. No additional handling time was incurred because this step replaced the now superfluous overwrap procedure.

Unexpectedly, visible microaggregates were found in 25% of the microwave-

thawed units. No common causative factor was discovered though several possibilities, such as microwave thawing position and exposure interval, were investigated and discounted. A similar instance of discoloration and aggregation of RBC occurring in a microwave blood warmer has been reported.[18] Studies by Slater reported no visible change in appearance of frozen RBC when they were microwave thawed while suspended in a heat dispersing media.[35]

Elevated WBC counts (Table 4) and morphological anomalies also occurred in the units with visibly abnormal appearances. It was felt that the aggregates were not lysed sufficiently rapidly to prevent their incorrect enumeration as WBC in the automated cycle of the test instrument. The presence of abnormal cell morphology suggested that local areas of overheating occurred in these units. Similar morphological phenomena have been documented to occur in whole blood heated to temperatures above 49 °C[57] or in saline-suspended cells heated above 37 °C.[58] Core temperatures recorded in the present study, with 2 exceptions, were below these temperatures and no significant difference between the experimental thaw groups was observed (Table 2). However, because each unit was mixed before the core temperature was recorded a small quantity of the blood in each unit may have been overheated without detection. The unchanged osmotic fragilities observed (Figure 3) suggested that, if this occurred, only a small percentage of cells were involved. This supposition is supported by earlier studies reporting stable osmotic fragilities at the temperatures observed in the present study.[16,34,50,59,60]

The high temperatures reached by 2 of the microwave-thawed units exceeded

the AABB standard[1,2] for all RBC preparations. This standard requires that RBC not be heated to above 38 °C. This standard does not seem unnecessarily restrictive, because as already noted, brief exposure of saline-suspended RBC to temperatures above 37 °C caused observable cell damage.[57-60]

Under the most ideal conditions, the thawing time for a frozen RBC unit has been reported to be at least 30 min[3,4] although 1 researcher cited the seemingly unrealistic average unit thaw time of 10 min.[46,49,61] The present study found that a significant percentage of control units (30%) did not reach an acceptable deglycerolization temperature even after the maximum allowable 40 min waterbath immersion. In fact, waterbath-thawed units often required additional RT incubation (Table 1). This under-estimation of conventional thawing time makes microwave processing an even more attractive proposition. In the present study, mean savings in thaw time of 80% was realized using microwave processing. This equated to savings ranging from 27-65 min.

The wide range of microwave thaw times for equal-sized units was puzzling (Table 1). One explanation involved the position of the unit relative to the point of origin of the electromagnetic wave within the chamber of the microwave oven. This was discounted when no consistent pattern was evident when comparing the 4 possible positions of the bag-holder in the microwave and their individual thawing times. A more feasible, but untested, explanation involved a variation in the final water and glycerol content in the frozen RBC.[62-65] Small differences in glycerolization would not impair function or recovery of the surviving cells but would affect microwave

absorptivity. Variation in thaw time could be attributed to either residual water retention by the cells during glycerolization or to differences in mixing because of small variations in hematocrit.[62] Because the highly polar water molecule is the primary action point of microwave energy, and because microwave heating is proportional to water content,[6] any difference in content would effect thaw rate. The high dielectric constants of water, glycerol, and protein all contribute to high microwave absorbing capacity and could vary slightly in each unit.

It was interesting to note that 3 overwrapped waterbath-thawed units developed leaks and allowed direct contact between water in the bath and the blood bags. These units required no additional RT incubation and were the only waterbath units to exceed core temperatures of 30 °C. This confirmed the decrease in thaw time expected without the overwrap interface but unacceptably exposed the units to bacterial contamination. Similar decreased thaw times for unwrapped frozen blood products have been recorded in previous studies.[3,66,67]

Red blood cell recovery was unpredictable regardless of thaw method and even conventional waterbath processing did not ensure the required 80% recovery (Table 2). Mean NBRL recoveries of approximately 90% correlated well with those seen in a previous study.[61] It was noted that recovery of microwave-thawed RBC performed at least as well as waterbath-thawed RBC.

Both supernatant HGB and potassium concentrations for all RBC units tested increased markedly from immediately post-wash through the 72 h storage interval (Fig 1,2). The increase in concentrations conformed with those reported by Valeri.[61]

Microwave-thawed RBC units exhibited significantly higher concentrations of supernatant HGB initially, which increased to more elevated levels at the end of 72 h of storage. No significant difference in potassium values were noted between the 2 thaw groups. Radioactive marker tests were not available to differentiate between active and passive transport mechanisms involved in the ion exchange. Previous studies have related microwave exposure to a decrease in active cation transport and an increase in passive diffusion associated with membrane phase transition.[31] Ham[7] and Staples[12] postulated that improper heating of RBC resulted not only in overt hemolysis but also produced a population of damaged but intact RBC with shortened survival. Such a result would be seen as a continual and accelerated lysis throughout the shelf-life of the product. McCullough[13] also theorized that the observed elevation in concentrations of HGB and potassium may be related to microwave-induced damage producing RBC membrane disruption with subsequent leakage and hemolysis.

After 72 h, both control and microwave RBC units exceeded the AABB guideline for supernatant HGB concentration of less than 200 mg/dL.[1,2] Waterbath-thawed units had acceptable transfusion levels through 48 h of storage achieving a mean value of 195 mg/dL while, after this same period, microwave-thawed units averaged an unacceptable 222 mg/dL. However, if the current guidelines for transfusion within 24 h post-deglycerolization[1,2,36] were followed, all units would have been acceptable for transfusion throughout the mandated 24 h maximum storage interval.

No changes in CBC parameters, except MCV, were evident over the

monitoring period (Table 3). Measurement of RBC indices in control and microwave RBC units showed a progressive increase in MCV over time which was attributed to the decreasing pH and DPG levels. As DPG decreased, chloride would move into the cell to maintain electroneutrality. Water would accompany this inward movement of chloride and would produce the cellular swelling noted. Cellular acidification from metabolic waste products such as lactate would also produce a decrease in net negative charge of the intracellular solutes. Again, to maintain electroneutrality, chloride and water would move into the cell and cause swelling. These reactions have been previously documented by Jacobs.[57]

That ATP measurements reflect the red cell membrane integrity, and thus post-transfusion survival, has been documented.[43-52] The absence of a significant difference in ATP levels in the 2 experimental thaw groups suggested that no appreciable alteration in RBC metabolic function resulted from microwave thawing (Figure 4). Peck[43] reported that ATP concentrations greater than 40% of starting levels provided RBC viability greater than the required 70%.[1,2] Overall, the ATP levels obtained on all units, whether waterbath-thawed or microwave-thawed, were greater than 40% of the starting levels throughout the 72 h interval (Figure 4B).

Both thaw groups in the present study exhibited ATP concentrations elevated above prefreeze values implying an alteration in normal homeostatic control. Further, ATP concentrations decreased more slowly in the RBC exposed to microwave thawing than in the waterbath control RBC. This paralleled a report by Fisher who documented a similar elevation in ATP in microwave-warmed RBC.[32] The elevated

ATP levels noted in the present study were contrary to past reports that both ATP and DPG were unaffected by the freezing process[52] but agreed with reports that found the phosphate in the post-thaw wash solution caused elevated ATP during subsequent storage of the cells.[68,69] Linko *et al.* reported a tendency for ATP and DPG to decrease after prolonged waterbath immersion but found that there was a tendency for both to increase following electromagnetic warming. They concluded that the radiation appeared to influence RBC metabolism in a manner not fully explained by temperature alone.[17]

The DPG concentration immediately post-thaw reflected prefreeze levels. The discrepancy between the past and present research with regard to this measure may reflect the fact that, in past studies, the immersion times varied widely for waterbath units and exceeded 60 min in the study by Linko.[17] Maintenance of DPG concentrations is associated with the ability of hemoglobin to capture, transport, and release oxygen.[46-52,54] Since the purpose of most blood transfusions is to supply viable RBC to a deficient recipient and thus to increase the capacity for oxygen transport, any manipulation negatively impacting on normal recovery must be avoided. In the present investigation, DPG concentration in microwave-thawed RBC were not significantly different from those seen in the waterbath-thawed cells and both showed a rapid decline post-thaw (Figure 5). A contributing factor to the rapid decrease in DPG concentrations was the presence of chloride in the wash solution which has previously been associated with depressed DPG.[69] The loss of DPG noted in both treatment groups could also be a side effect of the decreased pH.[70]

Overall, the microwave-thawed RBC units compared favorably with those units thawed by conventional waterbath method indicating that functional and biologic integrity of the blood was not altered by the electromagnetic radiation. Significant differences were noted only in supernatant HGB levels and WBC counts. However, the increase in supernatant HGB was not clinically significant at the approved life span of the processed product and would be of even less concern in that the majority of supernatant would be removed prior to transfusion. This conclusion would not hold for military contingencies where deglycerolized RBC may be transfused up to 3 days post-thaw. The presence of microaggregates in 25% of the microwave-thawed RBC units was of concern. These aggregates could promote deleterious pulmonary side effects if not removed by standard filtration. The elevation of ATP levels may reflect metabolic stimulation beneficial to prolonged cell survival and function. Significant savings in processing time were realized when the modified microwave oven was used to thaw frozen RBC. However, the treatment was not predictable with regard to the length of processing time and core temperature resulting from electromagnetic exposure.

REFERENCES

1. Standards for Blood Banks and Transfusion Services, 13th edition. Ed by Holland PV. American Association of Blood Banks, Arlington, VA, 1989.

2. Technical Manual, 10th edition. Ed by Walker RH. American Association of Blood Banks, Arlington, VA, 1990.

3. Smith DA, Monaghan WP, Orcutt RM, Barracchini AE, Valeri CR. A pre-sealed overwrap method of protecting frozen blood components during water immersion thawing. Transfusion 21(4):447-449, 1981.

4. Standard Operating Procedure: Red blood cells collected in the CPDA-1 800 mL primary PVC plastic bag collection system and stored for 3 to 6 days prior to glycerolization in the primary 800 mL bag with the special adaptor Port using 40% glycerol and storage at -80 °C, washed in the Haemonetics Blood Processor 115, and stored at 4 °C for 24 hours prior to transfusion. Naval Blood Research Laboratory/Boston University School of Medicine. Boston, MA, 1991.

5. Military Blood Program, 2004 Master Concept Plan. Armed Services Blood Program Office. Department of Defense, 1985.

6. Osepchuk, JM. Sources and basic characteristics of microwave/radio frequency radiation. Bulletin of the New York Academy of Medicine 55(11):976-998, 1979.

7. Ham TH, Shen SC, Fleming EM, Castle WB. Studies on the destruction of red blood cells. IV. Thermal injury: action of heat in causing increased spheroidicity, osmotic and mechanical fragilities and hemolysis of erythrocytes; observations on the mechanism of destruction of such erythrocytes in dogs and in a patient with a fatal thermal burn. Blood 3:373-403, 1948.

8. Restall CJ, Leonard PF, Taswell HF, Holaday RE. A microwave blood warmer: preliminary report. Anesthesia and Analgesia 45(5):625-628, 1967.

9. duPlessis JME, Bull AB, Besseling JLN. An assessment of radio frequency induction heating of blood for massive transfusion. Anesthesia and Analgesia 46(1):96-100, 1967.

10. Arens JF, Leonard GL. Danger of overwarming blood by microwave. Journal of the American Medical Association 218(7):1045-1046, 1971.

11. Leonard PF, Restall CJ, Taswell HF, Fairbanks VF. Microwave warming of bank blood. Anesthesia and Analgesia 50(2):302-305, 1971.

12. Staples PJ, Griner PF. Extracorporeal hemolysis of blood in a microwave blood warmer. The New England Journal of Medicine 285(6):317-319, 1971.

13. McCullough J, Polesky HF, Nelson C, Hoff T. Iatrogenic hemolysis: A complication of blood warmed by a microwave device. Anesthesia and Analgesia 51(1):102-106, 1972.

14. Dalili H, Adriani J. Effects of various blood warmers on the components of bank blood. Anesthesia and Analgesia 53(1):125-131, 1974.

15. Dalili H, Adriani J, Wu WT, Samuels MS. Radiowave and microwave blood warmers: Comparison with water bath blood warming units. Southern Medical Journal 66(11):1254-1259, 1973.

16. Linko K, Hynynen K. Erythrocyte damage caused by the Haemotherm microwave blood warmer. Acta Anaesthesiologica Scandinavica 23:320-328, 1979.

17. Linko K, Hekali R. Influence of the Taurus radiowave blood warmer on human red cells: Hemolysis and erythrocyte ATP and 2,3 DPG concentrations following warming by radiowaves, microwaves and water bath. Acta Anaesthesiologica Scandinavica 24:46-52, 1980.

18. Leaman PL, Martyak GG. Microwave warming of resuscitation fluids. Annals of Emergency Medicine 14(9):876-879, 1985.

19. Iserson KV, Huestis DW. Blood warming: current applications and techniques. Transfusion 31(6):558-569, 1991.

20. Roth-Henschker H, Holzberg E, Oppitz KH, Lehmann C. Warming of resuspended packed red cells with a new microwave device. Anaesthesist 37:321-330, 1988.

21. Mead JH, Boucock BP, Russell RE, Robinson CS, Harris G. Water environment microwave thawing. American Journal of Clinical Pathology 85(4):510-513, 1986.

22. Checcucci A, Benelli G, Duminuco M, Gaetani ML, Paoletti P, Vannini S, Morfini M. Reliability of microwave heating for hemoderivative thawing. Journal of Microwave Power 18(2):163-168, 1983.

23. Rock G, Tackaberry ES, Dunn JG, Kashyap S. Rapid controlled thawing of fresh-frozen plasma in a modified microwave oven. Transfusion 24(1):60-65, 1984.

24. Sherman LA, Dorner IM. A new rapid method for thawing fresh frozen plasma. Transfusion 14(6):595-597, 1974.

25. Thompson KS, O'Kell RT. Comparison of fresh-frozen plasmas thawed in a microwave oven and in a 37 °C water bath. American Journal of Clinical Pathology 75(6):851-853, 1981.

26. Westphal RG, Tindle B, Howard PL, Golden EA, Page GA. Rapid thawing of fresh frozen plasma. American Journal of Clinical Pathology 78(2):220-222, 1982.

27. Hurst TS, Turrentine MA, Johnson GS. Evaluation of microwave-thawed canine plasma for transfusion. Journal of American Veterinary Medicine Association 190(7): 863-865, 1987.

28. Sohngen D, Kretschmer V, Franke K, Pelzer H, Walker WH. Thawing of fresh-frozen plasma with a new microwave oven. Transfusion 28(6):576-580, 1988.

29. Luff RD, Kessler CM, Bell WR. Microwave technology for the rapid thawing of frozen blood components. American Journal of Clinical Pathology 83(1): 59-64, 1985.

30. Operation of the WesLabs Plasma Defroster. Westmorland Laboratories Inc, Sackville, New Brunswick, Canada, 1989.

31. Olcerst RB, Belman S, Eisenbud M, Mumford WW, Rabinowitz JR. The increased passive efflux of sodium and rubidium from rabbit erythrocytes by microwave radiation. Radiation Research 82:244-256, 1980.

32. Fisher PD, Poznansky MJ, Voss WAG. Effect of microwave radiation (2450 MHz) on the active and passive components of $^{24}Na^{+}$ efflux from human erythrocytes. Radiation Research 92:411-422, 1982.

33. Dunscombe PB, Gammampila K, Ramsey NW. A search for non-thermal effects of 434 MHz microwave radiation on whole human blood. Radiation Research 96:235-250, 1983.

34. Hamrick PE, Zinkl JG. Exposure of rabbit erythrocytes to microwave radiation. Radiation Research 62:164-168, 1975.

35. Slater RA. An Alternative Method of Thawing Frozen Blood Products Using Microwave Technology. Master Thesis for Bowling Green State University, Bowling Green, OH, 1989.

36. Code of Federal Regulation. Title 21, Parts 600-799. US Government Printing Office, Washington, D.C., Current edition.

37. Technical Insert, Sickle Cell Reagent Set. Baxter Healthcare Corportation, Miami, FL, 1987.

38. User's Guide, The Advanced Model 3DII Osmometer. Advanced Instruments, Inc., Needham Heights, MA II:3-5, 1991.

39. Technical Insert, Quantitative Colorimetric Determination of Hemoglobin in Whole Blood. Stanbio Laboratory, Inc., San Antonio, TX, 1989.

40. Operating and Service Instructions for the Astra Automated STAT/Routine Analyzer Systems: Sodium-Potassium Chemistry Module. Astra 8 System, Fullerton, CA 1:Sections 1-1 through 4-4, 1987.

41. User's Guide, The Coulter S-Plus III Series Instrument. Coulter Electronics, Hialeah, FL 1:Sections 1-1 through 2-22, 1988.

42. Laboratory Methods in Hematology and Hemostasis, Clinical Hematology and Fundamentals of Hemostasis. Ed by Harmening-Pittiglio D, Sacher RA. FA Davis Company, Philadelphia, PA 31:425-428, 1987.

43. Peck CC, Moore GL, Bolin RB. Adenine in blood preservation. Critical Reviews in Clinical Laboratory Sciences 13:173-212, 1981.

44. Hillyer CD, Tiegerman KO, Berkman EM. Evaluation of the red cell storage lesion after irradiation in filtered packed red cell units. Transfusion 31(6): 497-499, 1991.

45. Dern RJ, Brewer GJ, Wiorkowski JJ. Studies on the preservation of human blood. II. The relationship of erythrocyte adenosine triphosphate levels and other in vitro measures to red cell storageability. Journal of Laboratory and Clinical Medicine 69(6):968-978, 1967.

46. Valeri CR, Zaroulis CG. Rejuvenation and freezing of outdated stored human red cells. The New England Journal of Medicine 287(26):1307-1313, 1972.

47. Barlett GR. Red Cell Metabolism: Review Highlighting Changes During Storage. Ed by Greenwalt TJ, Jamieson GA. Grune and Stratton, New York, NY 1:5-24, 1973.

48. Weed RI, LaCelle PL, Udkow M. Structure and Function of the Red Cell Membrane: Changes During Storage. Ed by Greenwalt TJ, Jamieson GA. Grune and Stratton, New York, NY 4:65-81, 1973.

49. Valeri CR. Factors influencing the 24-hour posttransfusion survival and the oxygen transport function of previously frozen red cells preserved with 40 per cent W/V glycerol and frozen at -80 °C. Transfusion 14(1):1-15, 1974.

50. Myhre BA, Nakasako YY, Schott R. Studies on 4 °C stored frozen-reconstituted red blood cells: II. Chemical and cytological changes during storage. Transfusion 18(1):29-37, 1978.

51. Myhre BA, Nakasako YY, Schott R. Studies on 4 °C stored frozen-reconstituted red blood cells: III. Changes occurring in units which have been repeatedly frozen and thawed. Transfusion 18(2):199-203, 1978.

52. Moroff G, Dende D. Characterization of biochemical changes occurring during storage of red cells: Comparative studies with CPD and CPDA-1 anticoagulant-preservative solutions. Transfusion 23(6):484-489, 1983.

53. Technical Insert, Adenosine 5'-Triphosphate. Sigma Chemical Company, St Louis, MO, 1985.

54. Duhm J, Gerlach E. Metabolism and Function of 2,3-Diphosphoglycerate in Red Blood Cells. Ed by Greenwalt TJ, Jamieson GA. Grune and Stratton, New York, NY 6:110-139, 1973.

55. Technical Insert, 2,3-Diphosphoglyceric Acid. Sigma Chemical Company, St Louis, MO, 1985.

56. Minitab Reference Manual. Minitab Statistical Software Release Numbers 7.1 and 8.0, Minitab Inc., State College, PA. April 1989.

57. Jacobs MH, Stewart DR. Osmotic properties of the erythrocyte: Ionic and osmotic equilibria with complex external solution. Journal of Cellular Comparative Physiology 30:79-103, 1947.

58. Checcucci A, Olmi R, Vanni R. Thermal haemolytic threshold of human erythrocytes. Journal of Microwave Power 18(2):161-163, 1985.

59. VanderWalt JH, Russell WJ. Effect of heating on the osmotic fragility of stored blood. British Journal of Anaesthesia 50:815-820, 1978.

60. Rakow AL, Hochmuth RM. Effect of heat treatment on the elasticity of human erythrocyte membrane. Biophysiology Journal 15:1095-1100, 1975.

61. Valeri CR, Valeri DA, Anastasi J, Vecchione JJ, Dennis RC, Emerson CP. Freezing in the primary polyvinyl-chloride plastic collection bag: A new system for preparing and freezing nonrejuvenated and rejuvenated red blood cells. Transfusion 21(2):138-149, 1981.

62. Meryman HT. The cryopreservation of blood cells for clinical use. Progress in Hematology 11:193-227, 1979.

63. Mazur P. Theoretical and experimental effects of cooling and warming velocity on the survival of frozen and thawed cells. Cryobiology 2(4):181-192, 1966.

64. Pegg DE, Diaper MP, Skaer HLeB, Hunt CJ. The effect of cooling rate and warming rate on the packing effect in human erythrocytes frozen and thawed in the presence of 2 M glycerol. Cryobiology 21:491-502, 1984.

65. Marsland TP, Evans S, Pegg DE. Dielectric measurements for the design of an electromagnetic rewarming system. Cryobiology 24:311-323, 1987.

66. Rhame FS, McCullough J. Nosocomial *Pseudomonas* septicemia infection. Morbidity and Mortality Weekly Report 28:289-290, 1979.

67. Plotz RD, Ciotola RT. Thawing of fresh-frozen plasma at 45 °C versus 37 °C: Comparison using satellite packs of the same donor units. American Journal of Clinical Pathology 89(3):381-384, 1988.

68. Beutler E, Duron O. The preservation of ATP: The effect of phosphate. Transfusion 6(1):124-129, 1966.

69. Meryman HT, Hornblower M, Keegan T, Syring R, Heaton A, Mesbah-Karimi N, Bross J. Refrigerated storage of washed red cells. Vox Sanguinis 60:88-98, 1991.

70. Dawson RB, Kocholaty WF, Gray JL. Hemoglobin fuction and 2,3-DPG levels of blood stored at 4 °C in ACD and CPD: pH effect. Transfusion 10(6): 299-304, 1970.

71. User's Guide, Perkin Elmer Lambda Array UV/Visible 3840 Spectrophotometer. Perkin Elmer, Norwalk, CT. 1990.

APPENDIX A. TEST METHODOLOGY

1. Sickle Dex

Red blood cells from persons with sickle cell trait or disease will tolerate glycerolization but will hemolyze during deglycerolization. This characteristic would artificially skew the test model under investigation for any pool containing such a unit. Accordingly, all RBC units used were screened for the presence of hemoglobin S (HgbS) prior to pooling and, if positive, were excluded. Sickle cell screening was accomplished using a commercial kit (Baxter Healthcare Corporation, Miami, FL).[37]

The principle of the test involved the lysis of RBC by saponin to free the cellular HGB. The released HGB was then reduced by sodium dithionite in a phosphate buffer base. Reduced HgbS was characterized by its low solubility in the buffer and persistent turbidity was seen. In contrast, normal HGB exhibited no visible turbidity.

Two mL volumes (serological pipette, Costar Corporation, Cambridge, MA) of sickle cell reagent were placed in labeled 12x75 mm glass test tubes (Becton, Dickenson, & Company, Lincoln Park, NJ). A segment from each unit to be tested was obtained and mixed. Using an adjustable pipette (Medical Laboratory Automation, Inc., Mount Vernon, NY) with a disposable tip (Fisher Scientific, Pittsburgh, PA), 20 μL from each segment was added to the tubes containing the buffer. Pipette tips were changed for each sample. The test tubes were covered with parafilm (American Can Company, Greenwich, CT) and mixed by inversion. The

tubes were allowed to stand for 5 min at RT and then examined for evidence of turbidity. Known positive and negative cell controls (Baxter Healthcare Corporation, Miami, FL) were tested to monitor proper reagent function. Evidence of turbidity was reason for exclusion of the donor unit from the experimental pool.

2. Osmolality

Each RBC unit, waterbath- and microwave-thawed, had the osmolality of its supernatant determined immediately post-deglycerolization. Hyperosmolality in the supernatant (⟩500 mOsm) indicated inadequate glycerol removal and excluded the unit from the experimental pool. The exclusion was necessary to rule out the inhibitory effect on RBC function and survival associated with excessive glycerol retention.

The Model 3DII osmometer (Advanced Instruments, Inc., Needham Heights, MA)[38] was used to determine osmolality by freezing point depression. This instrument supercooled a 300 μL sample (Oxford Adjustable pipette and tips, Nichiryo Corporation, Japan) to below its freezing point and then vibrated the sample to induce ice crystal formation. The resulting heat of fusion elevated the temperature of the sample to its freezing point. This endpoint was determined by comparison to a standard calibration curve. The result was reported in milliosmols/kg (mOsm). A known 100 mOsm sodium chloride (NaCl) standard supplied by the manufacturer was assayed to monitor function of the instrument.

3. Waste and Supernatant Hemoglobin

The concentration of HGB in wash waste and in the supernatant of each unit

immediately post-wash, and after 24, 48, and 72 h of storage was tested using the cyanmethemoglobin technique.[39] Drabkin's reagent (Stanbio Laboratory, Inc., San Antonio, TX) was reconstituted in 2 L of purified deionized water (EI duPont deNemours & Company, Wilmington, DE) prior to use. All tests were performed in duplicate. A known whole blood control (Normal Control, Coulter Electronics, Hialeah, FL) was assayed to monitor proper reagent and instrument function. Pipettes (2, 5, and 10 mL sizes, Costar Corporation, Cambridge, MA) were used where appropriate. For sample sizes of 1 mL or less an adjustable pipette (Medical Laboratory Automation, Inc., Mount Vernon, NY) with a disposable tip (Fisher Scientific, Pittsburgh, PA) was used. Absorbance values were determined using the Lambda Array 3840 Spectrophotometer (Perkin Elmer, Norwalk, CT).[71]

Using Drabkin's reagent, HGB and it derivatives were oxidized by ferricyanide to methemoglobin. Cyanmethemoglobin was in turn formed from the methemoglobin by its reaction with cyanide. Formation of the complex was monitored by a peak absorbance ([A]) at 540 nm. The [A] obtained was directly proportional to the total HGB concentration in the sample.

The HGB concentration in the sample was calculated using a constant (K) derived from a calibration curve. The curve was established using varying concentrations of a known 18.0 grams per deciliter (g/dL) standard (Sigma Chemical Company, St Louis, MO). The working concentrations were produced by diluting the standard in Drabkin's reagent in the following manner:

Final HGB Concentration	Drabkin's Reagent(mL)	Standard(mL)
0.0 g/dL	6.0	0.0
4.5 g/dL	4.5	1.5
9.0 g/dL	3.0	3.0
13.5 g/dL	1.5	4.5
18.0 g/dL	0.0	6.0

During testing, the sample size was modified to adjust for the amount of HGB present in the waste and supernatant samples vs that present in whole blood samples. A conversion factor of 15.06 was included in calculations because of the modification (1:251 standard dilution vs 1:16.67 supernatant dilution).

The test procedure involved diluting 300 μL of the waste or supernatant in 4.7 mL of Drabkin's reagent (vs 20 μL in 5.0 mL for whole blood control) in a 10 mL tube (Superior Healthcare Group Inc., Cumberland, RI). The tube was capped, mixed by inversion, and allowed to stand for at least 3 min. The mixture was then transferred to a 5.0 mL cuvette (Columbia Diagnostics, Inc., Springfield, VA) and the [A] at 540 nm determined. The spectrophotometer was adjusted to zero with Drabkin's reagent.

Absorbance values were used to calculate HGB levels using the following equations:

$$\text{Supernatant Hemoglobin (mg/dL)} = \frac{K \times \text{Sample[A]}}{15.06} \times 1000$$

$$\text{Waste Hemoglobin (g)} = \frac{\text{mg/dL Hemoglobin} \times \text{Waste Volume (mL)}}{100{,}000}$$

4. Supernatant Potassium

Supernatant potassium concentrations were determined for each RBC unit immediately post-deglycerolization and after 24, 48, and 72 h of storage. An Astra Automated STAT-Routine Analyzer (Astra 8 System, Fullerton, CA)[40] was used. Concentrations were calculated directly against the manufacturer's known calibration standard. A 50 μL specimen was automatically sampled by the instrument.

The test module utilized an ion-exchange membrane to determine the level of potassium present in a buffered solution. The cavities of the membrane system roughly equalled the diameter of the potassium ion. When an ion exchange occurred, a voltage change took place within the membrane and was measured by voltage-sensitive circuits within the system.

5. Complete Blood Count

Each unit was tested to establish a prefreeze baseline CBC and a CBC at the time of the wash and after 24, 48, and 72 h of storage. The automated Coulter S-Plus III (Coulter Electronics, Hialeah, FL)[41] was used. Manufacturer's tri-level controls monitored proper function of the instrument. Levels for RBC, WBC, HGB, HCT, and MCV were repeated in duplicate for each sample at each test interval. Instrument sample size was 200 μL; 1.6 μL for RBC/MCV determinations and 28.0 μL for WBC/HGB determinations.

The method used for cell counting and sizing was the detection and measurement of changes in electrical resistance produced when a cell, suspended in a conductive reagent, traversed a small aperture. The conductive reagent employed depended upon

the cell under investigation and lysed all unwanted cells. Hemoglobin concentration was determined photometrically against a blank-reference voltage retained in the analyzer. The MCV was calculated directly from the voltage pulse heights. The HCT was calculated from the RBC count and the MCV value.

The units for the various analytes measured were:

RBC ..... x 10^6 cells/μL
WBC ..... x 10^3 cells/μL
HGB ..... g/dL
HCT ..... Percent (%)
MCV ..... Femtoliter (fL)

6. Osmotic Fragility

Each experimental RBC unit was tested immediately post-wash and after 24, 48, and 72 h of storage to determine if the thawing protocol had induced membrane instability. Any instability would shift the hemolysis seen in the series of NaCl dilutions used to detect osmotic fragility.[42] The test also served as a measure of surface-to-volume ratio.

A series of dilutions was prepared from a 1% NaCl stock solution. This stock solution was made by suspending 5.0 g of NaCl crystals in 500 mL of deionized water. An analytical balance, a Grade 'A' volumetric flask (Lurex, Vineland, NJ), and purified deionized water (EI duPont deNemours & Company, Wilmington, DE) were used. The dilution series was generated weekly from the stock solution, stored at 2-6 °C, and warmed to RT before use. Serological pipettes (Costar Corporation, Cambridge, MA) in 2, 5, and 10 mL sizes along with a 100 mL graduated cylinder

(Lurex, Vineland, NJ) were used as needed to accomplish dilution steps. The concentration series were produced as follows:

NaCl%, Final Concentration	Stock % NaCl (mL)	Distilled Water (mL)
0.85	68.0	12.0
0.65	52.0	28.0
0.60	48.0	32.0
0.55	44.0	36.0
0.50	40.0	40.0
0.45	36.0	44.0
0.40	32.0	48.0
0.35	28.0	52.0
0.30	24.0	56.0
0.00	0.0	80.0

For each test, a series of ten 12x75 mm test tubes were labeled (0.85 through 0.00) and 2.5 mL of the appropriate NaCl concentration was added. A well-mixed 50 μL RBC sample was added to each of the 10 tubes. An adjustable pipette (Medical Laboratory Automation, Inc., Mount Vernon, NY) with a disposable tip (Fisher Scientific, Pittsburgh, PA) was used. A new pipette tip was used for each transfer. Each tube was covered with parafilm (American Can Company, Greenwich, CT) and mixed by inversion. A known normal control was processed in the same manner. The tubes were incubated at RT for 30 min, mixed gently, and centrifuged at 2000 rpm (920 x g) for 5 min using a calibrated Dynac II centrifuge (Becton, Dickenson, & Company, Lincoln Park, NJ).

After centrifugation, the supernatant of each tube was removed, placed in an ultraviolet semi-microcuvette (Fisher Scientific, Pittsburgh, PA), and its optical density (O.D.) read at 540 nm using the Lambda Array 3840 Spectrophotometer

(Perkin Elmer, Norwalk, CT).[71] The spectrophotometer was adjusted to zero using deionized water.

The percentage of hemolysis in each tube (x) was calculated using the following equation:

$$\% \text{ Hemolysis} = \frac{O.D._{(x)} - O.D._{(0.85\%)}}{O.D._{(0.00\%)} - O.D._{(0.85\%)}} \times 100$$

Hemolysis patterns for the waterbath-thawed units were compared to the patterns seen in the microwave-thawed units.

7. Adenosine 5'-Triphosphate

Adenosine 5'-triphosphate (ATP) concentrations were monitored as a means of predicting post-transfusion survival. Testing was done prefreeze, immediately post-wash and after 24, 48, and 72 h of storage. A quantitative analysis kit (Catalog #366-A, Sigma Diagnostics, St. Louis, MO)[53] measuring a series of enzymatic reactions was used. The Lambda Array 3840 Spectrophotometer (Perkin Elmer, Norwalk, CT)[71] recorded the reaction in disposable 5 mL polystyrene cuvettes (Columbia Diagnostics, Inc., Springfield, VA). Tests were performed in duplicate and a known standard (Catalog #A5384, Sigma Diagnostics, St. Louis, MO) monitored accuracy. Deionized water (EI duPont deNemours & Company, Wilmington, DE) was used in the test and to adjust the spectrophotometer to zero. Disposable 2, 5, and 10 mL serological pipettes (Costar Corporation, Cambridge, MA) and centrifuge tubes (Superior Healthcare Group Inc., Cumberland, RI) were used. For sample sizes of

1 mL or less an adjustable pipette (Medical Laboratory Automation, Inc., Mount Vernon, NY) with a disposable tip (Fisher Scientific, Pittsburgh, PA) was used.

The first reaction in the series, catalyzed by phosphoglycerate phosphokinase (PGK), converted ATP to adenosine diphosphate (ADP) and 1,3-diphosphoglycerate. The second reaction, catalyzed by glyceraldehyde phosphate dehydrogenase (GAPD), converted the resulting 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate while NADH was oxidized to nicotinamide adenine dinucleotide (NAD). This last reaction produced a spectrophotometrically measurable reduction in [A] at 340 nm.

A protein-free filtrate (PFF) was prepared for testing from each unit by mixing 1.0 mL of 12% trichloroacetic acid and 1.0 mL of the sample in a centrifuge tube. After 5 min on ice the sample was centrifuged at 3000 rpm (1380 x g) for 10 min (Dynac II, Becton, Dickenson, & Company, Lincoln Park, NJ). A 500 μL aliquot of the PFF was then mixed with 1.0 mL of buffered 3-phosphoglyceric acid and 1.5 mL deionized water in a 300 μg NADH vial. This mixture was transferred to a cuvette and the initial [A] at 340 nm recorded. Then, 40 μL of GAPD/PGK enzyme mixture (glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglyceric phosphokinase) was added, mixed, and the [A] recorded over a 10 min interval. The final [A] was the minimum [A] recorded during the 10 min interval.

Absorbance values were used to calculate the ATP level using the following equations:

Step 1. $\Delta[A]$ = Initial [A] - Final [A]

Step 2. Hemoglobin ATP (μmol/g) = $\dfrac{\Delta[A] \times 195}{\text{Unit HGB (g/dL)}}$

The factor 195 was derived as follows:

$$195 = \frac{3.04 \text{ X } 100}{6.22 \text{ X } 0.25}$$

3.04 = Volume (mL) of liquid in cuvette.
100 = Conversion of concentration per 1 mL to per dL.
6.22 = Millimolar absorptivity of NADH at 340 nm.
0.25 = Sample volume.

8. 2,3-Diphosphoglycerate

Diphosphoglycerate (DPG) concentrations were monitored as a means of predicting the oxygen-transport ability of the thawed RBC. Testing was done prefreeze, immediately post-wash and after 24, 48, and 72 h of storage. A quantitative analysis kit (Catalog #35-A, Sigma Diagnostics, St. Louis, MO)[52] measuring a series of enzymatic reactions was used. The Lambda Array 3840 Spectrophotometer (Perkin Elmer, Norwalk, CT)[71] recorded the reaction in disposable 5 mL polystyrene cuvettes (Columbia Diagnostics, Inc., Springfield, VA). Tests were performed in duplicate and a known standard (Catalog #53005, Sigma Diagnostics, St. Louis, MO) monitored accuracy. Deionized water (EI duPont deNemours & Company, Wilmington, DE) was used in the test and to adjust the spectrophotometer to zero. Disposable 2, 5, and 10 mL serological pipettes (Costar Corporation, Cambridge, MA) and centrifuge tubes (Superior Healthcare Group, Inc., Cumberland, RI) were used. For sample sizes of 1 mL or less an adjustable pipette (Medical Laboratory Automation, Inc., Mount Vernon, NY) with a disposable tip (Fisher Scientific, Pittsburgh, PA) was used.

A series of enzymatic reactions produced a measurable reduction in [A] at

340 nm. In a reaction catalyzed by 2,3-diphosphoglycerate phosphatase and 2-phosphoglycolic acid, DPG was hydrolyzed to 3-phosphoglycerate (3-PGA) and phosphorus. The 3-PGA then reacted with ATP in the presence of 3-phosphoglycerate phosphokinase to form 1,3-diphosphoglycerate (1,3-DPG) and ADP. The 1,3-DPG formed oxidized NADH to NAD which caused an observable reduction in [A].

A PFF was prepared from each unit by mixing 3.0 mL of 8% trichloroacetic acid and 1.0 mL of the sample in a centrifuge tube. After 5 min on ice the sample was centrifuged at 3000 rpm (1380 x g) for 10 min (Dynac II, Becton, Dickenson, & Company, Lincoln Park, NJ). A 250 μL aliquot of the PFF was then mixed with 2.5 mL NADH solution and 100 μL of ATP solution. Next, 20 μL of GAPD/PGK enzyme mixture and 20 μL of phosphoglycerate mutase were added. After an additional 5 min RT incubation, the initial [A] at 340 nm was recorded. In the final reaction step, 100 μL of phosphoglycolic acid solution was added and the entire mixture incubated for 30 min at RT. Final [A] was then recorded.

Absorbance values were used to calculate the DPG level using the following equations:

Step 1. Δ[A] = Initial [A] - Final [A]

Step 2. Corrected Δ[A] = Δ[A] - 0.030

The factor 0.030 corrected for the change in volume caused by the addition of the phosphoglycolic acid.

Step 3. Blood DPG (μmol/mL) = Corrected Δ[A] X 7.7

The factor 7.7 was derived as follows:

$$7.7 = \frac{2.99}{6.22 \text{ X } 0.0625}$$

2.99 = Volume (mL) of liquid in cuvette.
6.22 = Micromolar absorptivity of NADH at 340 nm.
0.0625 = Volume (mL) of original sample.

Step 4. $$\text{Hemoglobin DPG } (\mu\text{mol/g}) = \frac{\text{Blood DPG } (\mu\text{mol/mL}) \text{ X } 100}{\text{Hemoglobin (g/dL)}}$$

APPENDIX B. LIST OF ABBREVIATIONS

[A] . . . . . Absorbance

AABB . . . . . American Association of Blood Banks

ATP . . . . . Adenosine 5'-triphophate

CBC . . . . . Complete Blood Count

DPG 2,3-Diphosphoglycerate

FDA . . . . . Food and Drug Administration

fL . . . . . Femtoliter(s)

g . . . . . Gram(s)

g/dL . . . . . Grams per Deciliter

h . . . . . Hour(s)

HCT . . . . . Hematocrit

HGB . . . . . Hemoglobin

HgbS . . . . . Hemoglobin S

Hz . . . . . Hertz

K . . . . . Constant

Kg . . . . . Kilogram(s)

L . . . . . Liter(s)

MCV . . . . . Mean Cell Volume

mg . . . . . Milligram(s)

mg/dL . . . . . Milligram(s) per deciliter

MHz . . . . . Millihertz

min . . . . . Minute(s)

mL . . . . . Milliliter(s)

mm . . . . . Millimeter(s)

mOsm . . . . . Milliosmols/kg

NaCl . . . . . Sodium Chloride

NBS . . . . . National Bureau of Standards

nm . . . . . Nanometer(s)

NBRL . . . . . Naval Blood Research Laboratory

O.D. . . . . Optical Density

PFF . . . . . Protein-Free Filtrate

RBC . . . . . Red Blood Cell(s)

rpm . . . . . Revolutions per Minute

RT . . . . . Room Temperature

SD . . . . . Standard Deviation

SE . . . . . Standard Error

sec . . . . . Second(s)

vs . . . . . Versus

WBC . . . . . White Blood Cell(s)

μg . . . . . Microgram(s)

μL . . . . . Microliter(s)

μmol/g . . . . . Micromoles per Gram

Δ . . . . . Change

°C . . . . . Degrees Centigrade

% . . . . . .Percent

〉. . . . . Greater than

AN ASSESSMENT OF RAPID MICROWAVE THAWING FOR FROZEN RED BLOOD CELLS

Capt Dianne C. Davis
USAF, BSC, Blood Bank Fellow

The thawing of frozen red blood cells is currently regulated under standards overseen by the Food and Drug Administration (FDA) and the American Association of Blood Banks (AABB). The standard regarding frozen red cells requires thawing occur in either a waterbath or a dry-warmer environment maintained at 37 or 42 degrees Centigrade. Established during early freeze/thaw experiments, these protocols were set to insure recovery of at least 80 percent of the original red cell mass and a cellular viability 24-hour post transfusion of at least 70 percent. Adherence to these stipulations render existing thawing practices both time consuming and susceptible to covert bacterial contamination.

Currently, waterbath thawing can add up to 40 minutes to the processing time required to ready a frozen red blood cell unit for transfusion. This can prove life-threatening to patients in emergency situations. Further, the Military Blood Program Office's Program 2004 establishes prepositioned frozen blood products at strategic locations for wartime contingencies. Extended thawing times using waterbaths when the majority of available products are frozen significantly blunts the efficiency of wartime medical interventions. The thawing of large volumes of blood in short periods of time is a virtual impossibility.

This study plans to investigate the use of rapid microwave thawing in relation to time saved and its impact upon cell mass recovery and viability. Parallel studies will be undertaken in which pools of red cells will be frozen and thawed using the standard Naval Blood Research Laboratory (NBRL) protocol and varying only in the thawing environment. Comparative data will be collected on thaw time, final temperature, red cell recovery, supernatant hemoglobin, extracellular potassium, and viability measurements including osmotic fragility, adenosine 5'-triphosphate (ATP) and 2,3-diphosphoglycerate (DPG) levels. Evaluations will center on determining if microwave processing using a FDA-approved plasma defroster will negatively impact on cell membrane integrity.

The study's goal is to establish a rapid thawing protocol which could serve as an alternative to or replacement for the current waterbath protocols. If the microwave protocol proves equivalent in terms of the study's parameters, benefits will be apparent in terms of time savings per unit processed and will eliminate the threat of bacterial contamination present in the current waterbath thawing method.

Liquid blood designated excess by the National Naval Medical Center's Blood Bank will be used for this study. The final sample size will be approximately 40 units. ABO-compatible units will be pooled and divided into 250 mL portions. A complete cell count, ATP level, DPG level, and final volume by weight will be determined on each 250 mL unit. The method of freezing will follow the high glycerol, low temperature protocol endorsed by the Department of Defense for 800 mL storage bags as published by the NBRL. After

varying lengths of storage at or below -65 degrees Centigrade, the paired units will be thawed independently using either a 42 degree Centigrade waterbath or the FDA-approved microwave plasma defroster. When completely thawed, the unit will be removed, time noted, and internal temperature determined.

After deglycerolization each unit will be weighed and sampled immediately, at 24, 48, and 72 hours post thaw for the following tests: CBC, osmolality, osmotic fragility, supernatant hemoglobin, extracellular potassium, ATP, and 2,3-DPG. Red cell post-transfusion survival has been found to be related primarily to ATP level. Maintenance of oxygen-transport functions depend primarily on 2,3-DPG level. Freeze-Thaw-Wash recoveries will also be calculated.

ASSAYS

ATP (Adenosine-5'-triphosphate)

Levels will be determined using a kit for quantitative enzymatic determination. In a series of enzymatic reactions, NADH is oxidized to NAD causing a spectrophotometrically measurable reduction in absorbance at 340 nm. A standard of known ATP concentration will be ran with each test group to insure accurate methodology.

2,3-DPG (2,3-diphosphoglyceric acid)

Levels will be determined using a kit for quantitative enzymatic determination. In a series of enzymatic reactions, NADH is oxidized to NAD causing a spectrophotometrically measurable reduction in absorbance at 340 nm. A control of known DPG

concentration will be ran with each test group to insure accurate methodology.

Supernatant Hemoglobin

Supernatant hemoglobin levels will be measured with a direct spectrophotometric procedure.

Extracellular Potassium

Supernatant potassium levels will be measured directly by ion selective electrode against a known calibration standard.

CBC (Complete Blood Count)

The hemoglobin, hematocrit, and mean cell volume will be determined on a Coulter S-Plus III series instrument.

Freeze-Thaw-Wash Red Cell Recovery

The percent of red cell recovery post-deglycerolization will be calculated using two different accepted methods, AABB and NBRL.

Osmolality

Osmolality will be determined following deglycerolization as a quality control measure to insure adequate glycerol removal. Normal red cell function and post-transfusion survival are both a function of the adequacy of glycerol removal. The principle of freezing point depression will be used.

Osmotic Fragility

This test will be used to determine increased membrane leakage and instability induced by microwave treatment. Osmotic fragility will be determined by suspending the processed cells in various concentrations of sodium chloride. The red cells suspended in the hypotonic saline diluents will swell, become fragile, and at some

point will eventually lyse. The released hemoglobin serves as a marker of lysis and will be measured spectrophotometrically.

Statistical Analysis

Standard statistical analyses for paired sample studies will be used to analyze the data for significance of differences between the control waterbath-thawed group and the microwave-thawed group. Analysis of variance will be applied for calculation of the mean, standard deviation, and the confidence limits. Appropriate graphs and plots will be constructed to visually display data.

AN ASSESSMENT OF RAPID MICROWAVE THAWING FOR FROZEN RED BLOOD CELLS

A) ABSTRACT

The thawing of frozen red blood cells is currently regulated under standards overseen by the Food and Drug Administration (FDA) and the American Association of Blood Banks (AABB). The standard regarding frozen red cells requires thawing occur in either a waterbath or dry-warmer environment maintained at 37 degrees Centigrade. Established during early freeze/thaw experiments, these protocols were set to insure recovery of at least 80% of the original red cell mass and a cellular viability 24-hour post transfusion of at least 70%. Adherence to these stipulations rendered existing thawing practices both time consuming and susceptible to covert bacterial contamination. This study plans to investigate the use of rapid microwave thawing in relation to time saved and its impact upon cell mass recovery and viability. Parallel studies will be undertaken in which pools of red cells will be frozen and thawed using a standard Naval Blood Research Laboratory protocol (approved by the FDA) with variance only in the thawing environment. Comparative data will be collected on thaw time, final temperature, red cell recovery, supernatant hemoglobin, extracellular potassium, and viability measurements (osmotic fragility, adenosine 5'-triphosphate and 2,3-diphosphoglycerate levels). Evaluations will center on determining if microwave processing will negatively impact on cell membrane integrity. Successfully establishing this alternative methodology would show major benefits in time savings during routine or wartime application.

AN ASSESSMENT OF RAPID MICROWAVE THAWING
FOR FROZEN RED BLOOD CELLS

B) SPECIFIC AIMS

The proposed investigation is structured to assess the impact of rapid microwave thawing on frozen red blood cells. The goal is to establish a rapid thawing protocol which could serve as an alternative to or replacement for the current waterbath protocol. The investigational design centers on confirming equivalent red cell recovery and viability in microwave thawed cells verses waterbath thawed cells. If the microwave protocol proves equivalent in terms of the study's parameters, benefits will be apparent in terms of time savings per unit processed and will eliminate the threat of bacterial contamination present in the current waterbath thawing method.

C) BACKGROUND AND SIGNIFICANCE

The standard method established by blood bank regulatory agencies for thawing frozen red blood cells involves 37 degree Centigrade waterbath immersion (1-3). Additionally, current standard of care also includes an overwrapping of the blood storage bag in a sealed secondary pouch (4). The overwrap serves to limit possible exposure of the primary blood bag to bacterial contaminants inherent to all waterbath systems. Unfortunately this overwrap also creates an air-water interface detrimental to heat exchange and contributory to unacceptably extended thawing times.

The most significant benefit in using microwaves to thaw a frozen product is the rapidity at which the desired temperature is reached. Currently, waterbath thawing can add up to 40 minutes to the processing time required to ready a frozen red blood cell unit for transfusion (4). This can prove life-threatening to patients in emergency situations. Further, the Military Blood Program Office's Program 2004 establishes prepositioned frozen blood products at strategic locations for wartime contingencies (5). Extended thawing times using waterbaths when the majority of available products are frozen would significantly brunt the efficiency of wartime medical interventions. The thawing of large volumes of blood in short periods of time would be a virtual impossibility.

Recent studies have been conducted, primarily with fresh frozen plasma, to identify a alternative method of thawing frozen blood products using microwave technology (6-22). Efforts centered on significantly decreasing thaw times while maintaining the coagulation function of the plasma product. These studies have

proven microwave thawing of fresh frozen plasma is an acceptable alternative under specific circumstances. The data from these studies lead the FDA to approve a structurally modified microwave equipped with rotating chambers for plasma thawing (22, 23). This microwave will be used for the thawing of frozen cells in this research proposal.

Early 1940's studies by Ham on red cells concluded that heat affected the integrity of the membrane which resulted in changes in morphology. Also observed was an increase in osmotic fragility of the cells and in some instances gross hemolysis (24).

The majority of later microwave studies centered on providing warmed blood in surgical cases where large transfusion volumes were required (7, 11-18, 21). Though centered on heating blood from refrigerated storage temperature, not thawing from sub-zero temperatures, these studies are important in delineating possible effects of microwave treatment of red cells. Leonard et al. in 1971 concluded microwave radiation did not reduce cell viability. This conclusion was based on the absence of significant ATP variations between warmed and unwarmed units (17).

Subsequent warming studies determined, in some cases, increased hemolysis in microwaved units. This was seen in the form of increased supernatant hemoglobin. Investigators concluded uneven warming and high cell water content inhibited penetration of the electromagnetic energy (14). Later studies proved this effect could be avoided providing the unit was rotated properly within the heating field (25).

A study examining warming of red cells in a microwave modified with a rotating blood holder was conduct by Roth-Henschker et al. The modification allowed mixing to induce uniform warming and incorporated an automatic temperature monitoring device. Their conclusion was that microwave warming up to 32 degrees Centigrade with adequate mixing was safe as long as small volumes, hematocrits over 70% and increased viscosities were avoided (26).

However, none of these investigations specifically addressed frozen red cell thawing by microwave energy. Only limited information is available that suggests microwave energy can be applied to frozen red blood cell thawing. Slater examined the use of an unmodified carousel microwave and red cells immersed in a heat-dispersing medium such as sand, glycerol, and tap water. In this study units processed by microwave thawed five times faster than control units thawed in a waterbath. Red cell integrity was

well maintained as evidenced by stable ATP and 2,3-DPG levels. A clinically insignificant elevation of supernatant hemoglobin was demonstrated in the microwaved units (27).

This proposed study will further investigate the possibility of thawing frozen red blood cells using microwave energy. Modifications on previous studies include use of the single FDA-approved plasma microwave with modified mixing arms and automatic temperature sensing and cut-off devices. No heat-dispersing medium will be employed. Data will be accumulated to assess the time savings and membrane effects of the electromagnetic heat generation.

D) PRELIMINARY STUDIES

LCDR Slater's Master of Science thesis for the Graduate College of Bowling Green State University studied thawing of frozen blood products in a unmodified microwave as referenced above (27).

E) EXPERIMENTAL DESIGN

Liquid blood designated excess by the Blood Bank will be used for this study. The final sample size will be forty units. ABO-compatible units will be pooled and divided into 200 mL portions. A complete cell count (CBC) and final volume by weight will be determined on each 200 mL unit. The method of freezing will follow the high glycerol, low temperature protocol endorsed by the Department of Defense for 800 mL storage bags as published by the Naval Blood Research Laboratory (28). After varying lengths of storage at -80 degrees Centigrade, the paired units will be thawed independently using either a 37 degree Centigrade waterbath or the FDA-approved microwave plasma defroster. The thaw time for each unit will be recorded along with the internal unit temperature at the thawing endpoint.

The waterbath used will be a covered, thermostatically controlled, circulating waterbath with no more than one unit thawed at a time. Each unit will be enclosed, as accepted in standard practice, in a protective overwrap. The unit will be weighted to remain totally submerged during thawing and examined at five minute intervals. When completely thawed, the unit will be removed, time noted, and internal temperature determined. Maximum allowable waterbath immersion time will be 40 minutes as recommended by the freezing protocol. Microwave thawing will be accomplished in the

AN ASSESSMENT OF RAPID MICROWAVE THAWING
FOR FROZEN RED BLOOD CELLS

WesLabs Plasma Defroster manufactured by Westmorland Laboratories Inc. The microwave thawed units will not be overwrapped. Time interval to the thawed endpoint and the final internal temperature will be determined.

Sampling will be performed using a sterile plasma transfer system and aseptic technique. Samples will be maintained either on wet ice or in a 1-6 degree Centigrade refrigerator and tested immediately following aliquoting. Samples will be obtained pre-freeze, post-thaw, and post-deglycerolization. Further sampling will be done at 24, 48, and 72 hours.

Temperature measurements post-thaw will be taken using a YSI Tele-Thermometer with YSI 400 series probe. The probe will be inserted fully into the units immediately following removal from the thawing chamber. Any unusual physical appearance in the product following thawing will be noted. Thaw times will be measured using a calibrated laboratory timer.

After deglycerolization each unit will be weighed and sampled immediately, at 24, 48, and 72 hours post thaw for the following tests: CBC, osmolality, osmotic fragility, supernatant hemoglobin, extracellular potassium, ATP, and 2,3-DPG. Red cell post-transfusion survival has been found to be related primarily to ATP level. Maintenance of oxygen-transport functions depend primarily on 2,3-DPG level. Freeze-Thaw and Freeze-Thaw-Wash recoveries will also be calculated.

ASSAYS

ATP (Adenosine-5'-triphosphate)

Levels will be determined using a kit for quantitative enzymatic determination (Catalog #366-A, Sigma Diagnostics, St. Louis, MO.). In a series of enzymatic reactions, NADH is oxidized to NAD causing a spectrophotometrically measurable reduction in absorbance at 340 nm. A standard of known ATP concentration will be ran with each test group to insure accurate methodology (Catalog #A5394, Sigma Diagnostics, St. Louis, MO.). The Perkin Elmer Lambda Array 3840 Spectrophotometer will be used. Pre-freeze and post-deglycerolization measurements at time zero and 24, 48, and 72 hours will be made (29).

2,3-DPG (2,3-diphosphoglyceric acid)

Levels will be determined using a kit for quantitative enzymatic determination (Catalog #35-A, Sigma Diagnostics, St. Louis, MO.). In a series of enzymatic reactions, NADH is oxidized to NAD causing a spectrophotometrically measurable reduction in

absorbance at 340 nm. A control of known DPG concentration will be ran with each test group to insure accurate methodology (Catalog #53005, Metabolite Control, Sigma Diagnostics, St. Louis, MO.). The Perkin Elmer Lambda Array 3840 Spectrophotometer will be used. Pre-freeze and post-deglycerolization measurements at time zero and 24, 48, and 72 hours will be made (30).

Supernatant Hemoglobin

Plasma hemoglobin levels will be measured with a direct spectrophotometric procedure using the Perkin Elmer Lambda Array 3840 Spectrophotometer. Samples will be diluted 1:10 (0.3 mL of sample to 2.7 mL of Tris Buffer) and wavelength measurements taken at 450 nm, 415 nm, and 380 nm. Absorbance at 415 nm corresponds with the sample's hemoglobin concentration which will be corrected for triglyceride and bilirubin interferences at 380 nm and 450 nm respectively. Pre-freeze, post-thaw, and post-deglycerolization measurements at time zero and 24, 48, and 72 hours will be made.

Extracellular Potassium

Plasma potassium levels will be measured directly by ion selective electrode against a known calibration standard (Astra 8 System, Fullerton, CA.). Pre-freeze, post-thaw, and post-deglycerolization measurements at time zero and 24, 48, and 72 hours will be made.

CBC (Complete Blood Count)

The hemoglobin (HGB), hematocrit (HCT), and mean cell volume (MCV) will be determined on a Coulter S-Plus III series instrument. Pre-freeze and post-deglycerolization measurements at time zero and 24, 48, and 72 hours will be made.

Freeze-Thaw-Wash Red Cell Recovery

The percent of red cell recovery post-deglycerolization will be calculated using the formula: (Net weight (grams) of deglycerolized RBC X Hct) divided by (Net weight (grams) of pre-freeze RBC X Hct) times 100 (2).

Osmolality

Osmolality will be determined following deglycerolization as a quality control measure to insure adequate glycerol removal. Normal red cell function and post-transfusion survival are both a function of the adequacy of glycerol removal. The principle of freezing point depression on the Advanced Instruments Model 3DII will be used. A glycerol content of 1% produces osmolality of approximately 420 mOsm. Deglycerolized units with osmolality up to 500 mOsm apparently can be transfused without hemolysis and the range permissible by AABB standards is 420 to 500 mOsm. No unit exceeding 500 mOsm will be included in the final comparisons.

AN ASSESSMENT OF RAPID MICROWAVE THAWING
FOR FROZEN RED BLOOD CELLS

Osmotic Fragility

This test will be used to determine increased membrane leakage and instability induced by microwave treatment. Osmotic fragility will be determined by suspending the processed cells in various concentrations of sodium chloride (Catalog #5830, Becton-Dickinson, Rutherford, NJ.). The concentrations will vary from 0.00% to 0.85% NaCl. The red cells suspended in the hypotonic saline diluents will swell, become fragile, and at some point will eventually lyse. The released hemoglobin serves as a marker of lysis and will be measured spectrophotometrically using the Perkin Elmer Lambda Array 3840 Spectrophotometer (31).

F) Statistical Analysis

The paired Student's t-test and the Wilcoxon Rank-Sum test will be used to analyze the data for significance of differences between the control waterbath-thawed group and the microwave-thawed group. Analysis of variance will be applied for calculation of the mean, standard deviation, and the confidence limits. Appropriate graphs and plots will be constructed to visually display data.